MOLECULAR DETERMINANTS OF $GABA_A$ RECEPTOR DEGRADATION

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A thesis submitted to the University of London for the degree of Doctor of Philosophy

November 2006

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Abstract

γ-amino-butyric acid type A ($GABA_A$) receptors are the major sites of fast synaptic transmission in the central nervous system and can be assembled from 7 subunit classes: α1-6, β1-3, γ1-3, δ, ε, π, and θ. Although expression of receptor α and β subunits can produce functional GABA-gated chloride channels the presence of the γ2 subunit within the $GABA_A$ receptor complex has been shown to play a key role in clustering and synaptic targeting. The regulation of $GABA_A$ receptor cell surface stability by endocytosis has been previously shown to be a crucial determinant of inhibitory synaptic strength. The main focus of this thesis is to further understand the molecular mechanisms by which the number of $GABA_A$ receptors clustered in synapses can be regulated.

Microscopy and biochemical experiments showed that the γ2 subunit of $GABA_A$ receptors confers an enhancement in the targeting of $GABA_A$ receptors to a degradative pathway after internalization from the plasma membrane. Furthermore, blocking lysosomal degradation with the lysosomal inhibitor leupeptin results in increased GABAergic currents in cells expressing $GABA_A$ receptors containing α1, β3 and γ2 subunits but not those expressing α1β3 receptors alone. In order to characterise the molecular signals determining this degradative pathway, β3-γ2 chimeras were created and expressed in both HEK293 cells and hippocampal neurons. Quantitative confocal microscoscopy studies on these chimeras revealed a 20 amino acid region to be responsible for the late endosomal/lysosomal targeting of γ2 subunit containing $GABA_A$ receptors.

Further analysis into this 20 amino acid region identified a lysine stretch that when mutated resulted in decreased levels of internalised $GABA_A$ receptors in late endosomes. Within this region the mutation of serine 327, a known phosphorylation site of $GABA_A$ receptors, also caused a reduction in the targeting of internalised $GABA_A$ receptors to lysosomes. Interestingly GABAergic currents formed by the expression of α1β3 and γ2L (lysine or serine mutant) were

unaffected by the lysosomal inhibitor leupeptin. Biochemical studies found these lysines to be modified by the small poly-peptide ubiquitin, a known molecular signal for the degradation of proteins in the proteasome and the endocytic pathway. Moreover, mutation of serine 327 was sufficient to inhibit the ubiquitination of this lysine stretch in the intracellular loop of the γ2L subunit.

The results presented in this thesis suggest that the endocytic sorting fate of synaptic $GABA_A$ receptors plays an important role in determining the strength of inhibitory synapses and provide a mechanism by which serine 327 acts as a molecular switch to regulate the ubiquitination dependent degradation of $GABA_A$ receptors in the lysosome.

Ackowledgements

Despite the 3500 miles that separate the new Moss lab US and the old Moss lab UK, Steve has shown his constant support and enthusiasm for the work carried out here and described in this thesis. For this I am very grateful, as well as his guidance and constant challenging to help me develop as a scientist.

I would like to thank Mike Lumb for constructing the chimeras used in this thesis, Yuri Bogdanov and Richard Saliba for supplying constructs, and Cecilia Armstrong-Gold and Guido Michels for the electrophysiological data presented in this thesis.

I would also like to thank the past an present members of the Moss and Kittler labs who all contributed in my formation and made the lab a fun enviroment to work in. I am extremely grateful to the support I have received from Mike and Tony, who have helped me in many ways over the past four years.

I am also grateful to all my friends that have been there for the highs and lows of this PhD. In particular Liz, Jay and Alejandra who have been extremely patient and supportive. I am immensly grateful to Jo Kittler for his invaluable support both scientific and emotional over the past four years.

This thesis is dedicated to my parents and sisters.

Contents

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Abbreviations

ACh	Acetylcholine
AIS	Axon initial segment
AMPA	α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid
AP	Adaptor protein
APC	Anaphase promoting complex
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BiP	Immunoglobulin heavy chain binding protein
BSA	Bovine serum albumin
CamKII	Ca^{2+}/calmodulin dependent protein kinase II
CCV	Clathrin coated vesicle
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
COS	*Cercopithecus aethiops* (African green monkey kidney)
DIV	Days *in vitro*
DTT	Dithiothreitol
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethyleneadiaminetetraacetic acid
EGTA	Ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
et al	and others
GABA	γ-aminobutyric acid

$GABA_A$	γ-aminobutyric acid type A
$GABA_B$	γ-aminobutyric acid type B
$GABA_C$	γ-aminobutyric acid type C
GAD	Glutamic acid decarboxylase
GFP	Green fluorescent protein
GST	Glutathione S transferase
GTP	Guanosine triphosphate
HA	Hemagglutinin
HAP-1	Huntingtin associated protein 1
HBSS	Hank's buffered saline solution
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
IPSC	Inhibitory postsynaptic current
kDa	KiloDalton
LB	Luria broth
LTD	Long term depression
LTP	Long term potentiation
LUT	Look up table
MAP	Microtubule associated protein
MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
MVB	Multi-vesicular body
nAChR	Nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
NP-40	Nonylphenoxy polyethoxy ethanol

O.D	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIP2	Phosphtidylinositol 4,5 bisphosphate
PKA	cAMP depedent protein kinase
PKC	Ca2+/phospholipid dependent protein kinase
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleic acid
RNAi	RNA interference
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	Transmembrane
VIAAT	Vesicular inhibitory amino acid transporter

1. INTRODUCTION

Neuronal communication

Synaptic transmission describes the ability to receive, integrate and deliver signals between neuronal cells. The sites of communication between two cells are termed synapses of which two types exist: electrical and chemical. The majority of synaptic connections within the central nervous system use chemical transmission. Chemical synapses have a large (20-40nm) synaptic cleft into which a chemical neurotransmitter is released from the presynaptic terminal in response to an action potential (Fig. 1.1). Released neurotransmitter is able to diffuse across the synaptic cleft and bind specialized receptors on the postsynaptic membrane, which can respond to alter the probability of action potential firing. The manner of the response achieved by neurotransmitter binding to a post-synaptic receptor is determined by a number of factors including the amount and identity of neurotransmitter as well as the type and number of post-synaptic receptors. There are two major types of chemical synapses in the central nervous system (CNS): at excitatory synapses release of the neurotransmitter glutamate activates glutamate receptors resulting in a depolarization of the cell, increasing its chances of firing an action potential; whereas activation of GABA receptors at inhibitory synapses causes hyperpolarization moving the membrane potential away from the spike threshold for action potentials (Fig. 1.1).

GABA is the major inhibitory neurotransmitter in the brain

The majority of fast inhibitory neurotransmission in the adult central nervous system is mediated by the amino acid neurotransmitter γ amino butyric acid (GABA) acting on GABA type A ($GABA_A$) receptors. GABA is synthesized from glutamate in a reaction catalysed by glutamic acid decarboxylase (GAD). GABA was identified in large quantities in mammalian brain in 1950 (Roberts and Frankel, 1950; Udenfriend, 1950). The inhibitory neurotransmitter properties of GABA were beginning to be characterised in crustaceans throughout the mid 50s (Bazemore et al., 1956; Boistel and Fatt, 1958), although it wasn't until 1963 that

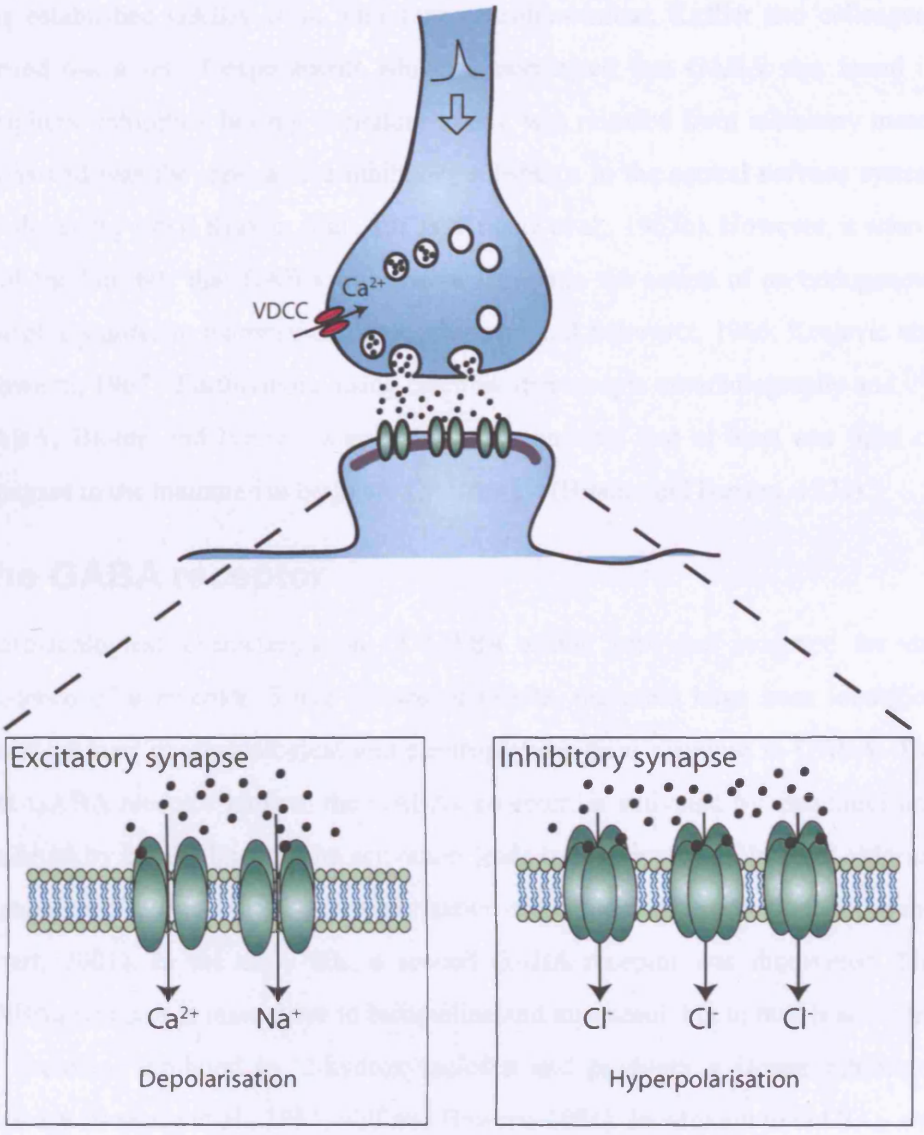


Figure 1.1: Neurotransmitter release at chemical synapses. Propagation of the action potential along the axon activates voltage-gated calcium channels (VDCC) which allow influx of calcium ions into the presynaptic terminal. An increase in calcium stimulates vesicle fusion with the plasma membrane allowing neurotransmitter release into the synaptic cleft and subsequent binding to receptors on the postsynaptic membrane. There are two main types of synapses: excitatory and inhibitory. Central excitatory synapses release the neurotransmitter glutamate, which binds to glutamate receptors allowing the influx of cations resulting in membrane depolarisation. In contrast activation of $GABA_A$ receptors at central inhibitory synapses results in hyperpolarisation due to an influx of chloride ions.

was established GABA as an inhibitory neurotransmitter. Kuffler and colleagues carried out a set of experiments which demonstrated that GABA was found in peripheral inhibitory but not excitatory axons, was released from inhibitory motor axons and was the most active inhibitory substance in the central nervous system (Dudel et al., 1963; Kravitz et al., 1963a; Kravitz et al., 1963b). However, it wasn't until the late 60s that GABA was shown to mimic the action of an endogenous neurotransmitter in mammalian brain (Krnjevic and Schwartz, 1966; Krnjevic and Schwartz, 1967). Furthermore, using electron microscopic autoradiography and [3H] GABA, Bloom and Iversen were able to demonstrate that at least one third of synapses in the mammalian brain are GABAergic (Bloom and Iversen, 1971).

The GABA receptor

Pharmacological characterization of GABA action provided evidence for the existence of a receptor. Three classes of GABA receptors have been identified based on their pharmacological and electrophysiological response to GABA. The first GABA receptor known, the $GABA_A$ receptor, is activated by muscimol and inhibited by bicuculline and its activation leads to opening of an integral chloride channel resulting in a rapid hyperpolarisation of the membrane potential (Moss and Smart, 2001). In the early 80s, a second GABA receptor was discovered. The $GABA_B$ receptor is insensitive to bicuculline and muscimol, but in turn is activated by baclofen, inhibited by 2-hydroxysaclofen and produces a slower inhibitory response (Bowery et al., 1981; Hill and Bowery, 1981). In addition to $GABA_A$ and $GABA_B$ receptors, a third receptor ($GABA_C$), homologous in structure to the $GABA_A$ receptor but insensitive to both bicuculline and baclofen and sensitive to the GABA analogue cis-4-aminocrotonic acid and picrotoxin has also been identified (Drew et al., 1984; Shimada et al., 1992; Quian and Dowling, 1993).

Pharmacology of the $GABA_A$ receptor

$GABA_A$ receptor function can be allosterically modulated by a range of therapeutic agents including benzodiazepines, barbiturate, steroids and anaesthetics (Macdonald and Olsen, 1994; Rabow et al., 1995; Fig.1.2). Benzodiazepines such

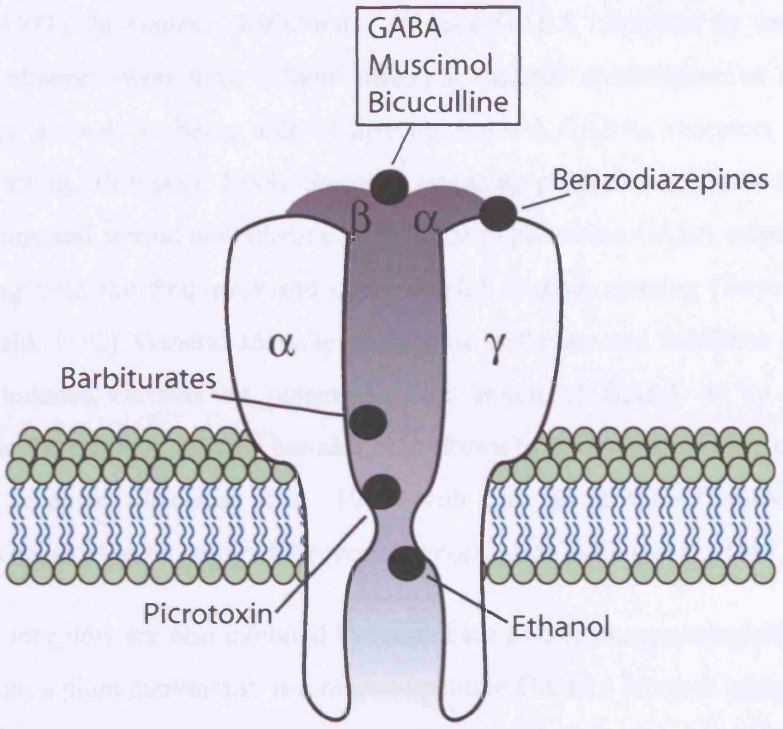


Figure 1.2: Site of action of pharmacological agents on $GABA_A$ receptors. The neurotransmitter GABA binds $GABA_A$ receptors on a site formed by the N-terminus of α and β subunits. $GABA_A$ receptors can be potentiated by binding of benzodiazepines to a site created at the interface of the α and γ subunit. In addition, $GABA_A$ receptors can also be modulated by barbiturates, ethanol and picrotoxin. The site of action for these drugs is believed to be inside the chloride permeable channel.

as valium and diazepam potentiate GABA responses by increasing the frequency of channel opening without altering channel open time or conductance (Study and Barker, 1981). In contrast, barbiturates enhance GABA responses by increasing average channel open time without affecting channel conductance or opening frequency as well as being able to directly activate $GABA_A$ receptors at high concentrations (Bormann, 1988). Steroids, including progesterone, corticosterone, testosterone and steroid anaesthetics are thought to potentiate GABA responses by increasing both the frequency and open time of channel opening (Twyman and Macdonald, 1992). General anaesthetics such as isoflurane and halothane increase GABA induced currents by potentiating the action of GABA or by directly activating the channel. Ethanol has also been shown to have a potentiating effect on $GABA_A$ receptors (Dietrich et al., 1989) with sites on receptor transmembrane domains believed to be required for receptor modulation (Mihic et al., 1997).

$GABA_A$ receptors are also inhibited by a number of other pharmacological agents. Picrotoxin, a plant convulsant, is a non competitive $GABA_A$ receptor inhibitor that is thought to act by binding to a site within the channel (Zhang et al., 1994) leading to stabilization of receptors in an agonist bound desensitised state (Newland and Cull-Candy, 1992). In addition, $GABA_A$ receptors lacking the γ subunit (and therefore extrasynaptic - see below) have been shown to be inhibited by zinc (Smart, 1992, Smart et al., 2004).

$GABA_A$ receptor structure

The $GABA_A$ receptor was initially purified using GABA/benzodiazepine affinity chromatography. SDS-PAGE analysis revealed two receptor subunits, α and β, with molecular weights of 53 kDa and 58 kDa respectively (Sigel, 1983; Sigel and Barnard, 1984). Oligonucleotides, designed based on peptide microsequencing results, were used for screening bovine cDNA libraries and allowed for the cloning of both α and β subunits. Analysis of the amino acid sequences of α and β subunits revealed that these receptors showed significant homology with each other and with the nicotinic acetylcholine receptor (nAChR).

$GABA_A$ receptors and nAChRs are members of the ligand gated ion channel superfamily, which are homologous in structure and include glycine receptors and serotonin (5HT3) receptors (Schofield et al., 1987; Unwin, 1993). Members of this family share a common subunit structure encompassing a large extracellular amino terminus, four transmembrane (TM) domains and a large intracellular loop between TM3 and TM4 (Fig. 1.3). In addition, based on biochemical and electron microscopy analysis members of this family have been shown to be pentameric in structure where subunits are arranged around a central aqueous pore (Unwin, 2003).

$GABA_A$ subunit diversity

Following the cloning of the α1 and β1 $GABA_A$ receptor subunits, a total of 16 genes encoding $GABA_A$ receptor subunits have been identified in the mammalian nervous system. Based on amino acid sequence homology five additional α subunits were identified (Levitan et al., 1988, Ymer et al., 1989; Khrestchatisky et al., 1989; Malherbe et al., 1990; Kato, 1990; Luddens et al., 1990) as well as another two β subunits (Ymer et al., 1989). Co-expression of α and β subunits in heterologous systems, however, formed receptors that lacked benzodiazepine sensitivity, a pharmacological trait observed in neuronal $GABA_A$ receptors. A novel $GABA_A$ receptor subunit that when co-expressed with α and β subunits inferred benzodiazepine sensitivity on the assembled receptors was then identified (Pritchett et al., 1989). This subunit, termed γ2, shows 42% and 35% identity to the α1 and β1 subunits respectively. Three γ subunits have been identified in total (Pritchett et al., 1989; Ymer et al., 1990; Wilson-Shaw et al., 1991). In addition to α, β and γ subunits other receptor subunits have been identified: δ (Shivers et al., 1989), ε (Davies et al., 1997; Whiting et al., 1997), π (Hedblom and Kirkness, 1997) and θ (Bonnert et al., 1999). In addition many of these genes undergo alternative splicing the most predominant of these being the γ2 subunit, which exists in a short (γ2S) and long (γ2L) form (Whiting et al., 1990; Kofuji et al., 1991).

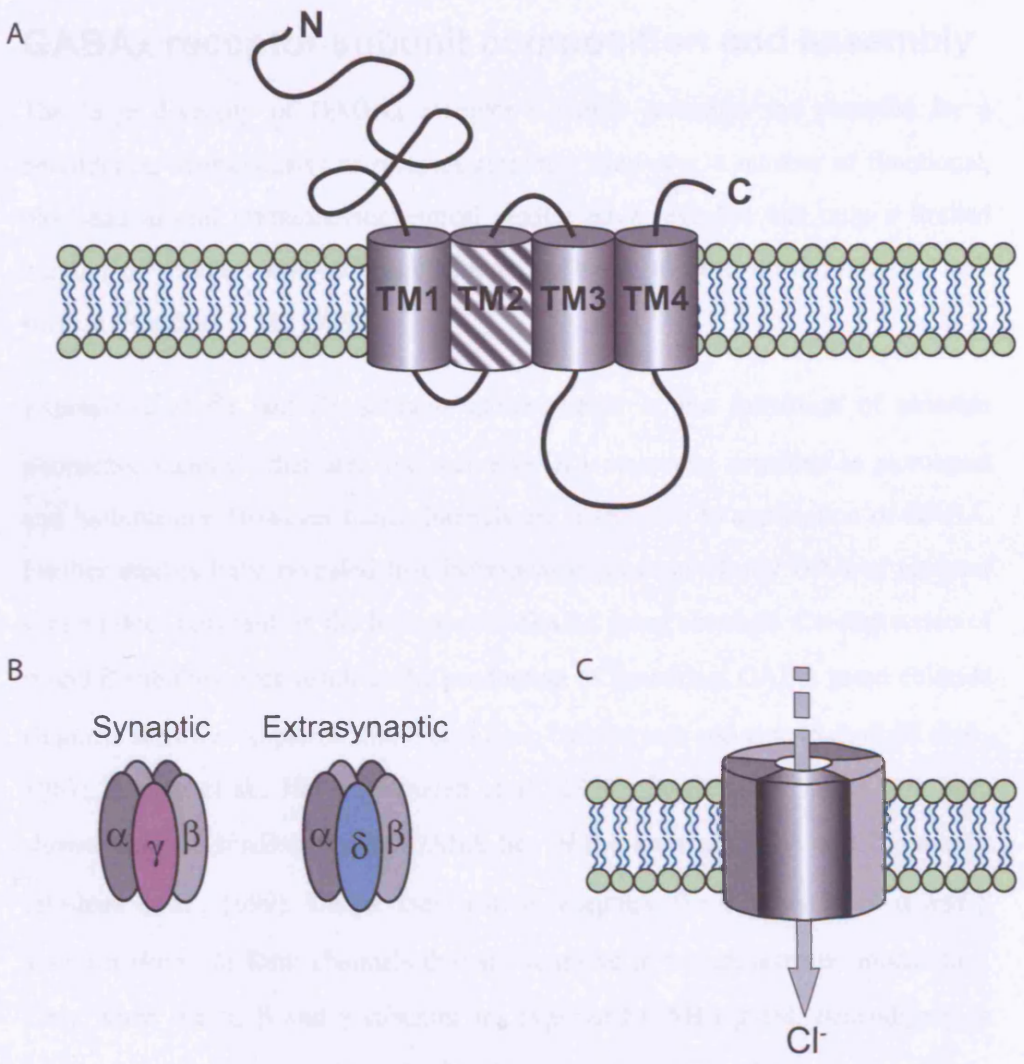


Figure 1.3: $GABA_A$ receptor structure. A. Transmembrane topology of the $GABA_A$ receptor. Receptor subunits consist of four hydrophobic transmembrane domains (TM 1-4) where TM2 is believed to line the pore of the channel. The large extracellular N-terminus is the site for ligand binding as well as the site of action of various drugs. Each receptor subunit also contains a large intracellular domain between TM3 and TM4, which is the site for various protein interactions as well as the site for post-translational modifications. B. Five subunits from seven subunit families assemble to form a heteropentameric channel. Despite the extensive heterogeneity of $GABA_A$ receptors, most synaptic receptors are thought to consist of 2α, 2β and 1γ subunit. Extrasynaptic receptors mainly consist of 2α, 2β and 1δ subunit. C. $GABA_A$ receptor subunits assemble to line a central chloride selective pore.

$GABA_A$ receptor subunit composition and assembly

The large diversity of $GABA_A$ receptor subunits generates the potential for a bewildering heterogeneity of receptor structure. However, a number of functional, biochemical and immunocytochemical studies have revealed that only a limited number of receptor subunit combinations are likely to exist on the neuronal cell surface (Sieghart et al., 1999).

Expression of β1 and β3 subunits alone results in the formation of chloride permeable channels that are, like native GABA receptors, sensitive to picrotoxin and barbiturates. However these channels are insensitive to application of GABA. Further studies have revealed that individual expression of any $GABA_A$ receptor subunit does not result in the formation of GABA gated channels. Co-expression of α and β subunits does result in the production of functional GABA gated chloride channels sensitive to picrotoxin, bicuculline, barbiturates and zinc (Schofield et al., 1987; Levitan et al., 1988, Draguuhn et al., 1990). Furthermore it has now been shown that the binding site of GABA lies in the interface of α and β subunits (Boileau et al., 1999). Unlike most native receptors, the expression of α and β subunits does not form channels that are sensitive to benzodiazepine modulation. Only when the α, β and γ subunits are expressed GABA gated, benzodiazepine sensitive channels are formed (Pritchett et al., 1989; Ymer et al., 1990). Interestingly these channels are insensitive to zinc modulation (Smart et al., 2004).

The above results together with biochemical experiments suggest that the most prevalent $GABA_A$ receptor subunit composition in the brain consists of α, β and γ subunits. In addition the δ, ε and θ subunits can substitute for the γ subunit to form benzodiazepine insensitive GABA gated channels (Quirk et al., 1994; Davies et al., 1997; Neelands and Macdonald, 1999; Shivers et al., 1989; Bonnert et al., 1999). Immunoprecipitation studies show that more than one α or β subunit isoform can coexist within the same receptor (McKernan et al., 1991; Mertens et al., 1993; Pollard et al., 1993; Khan et al., 1996) whereas most $GABA_A$ receptors are thought

to contain a single γ subunit (Mossier et al., 1994; Quirk et al., 1994). Furthermore, a number of studies suggest that the stoichiometry of these channels is of 2α, 2β and 1γ subunit (Chang et al., 1996; Farrar et al., 1999; Knight et al., 2000; Tretter et al., 1997).

Immunocytochemical analysis has revealed that only certain subunit combinations can access the cell surface whereas other combinations result in the subunits being retained in the endoplasmic reticulum (ER; Moss and Smart, 2001). These result suggest that there are molecular mechanisms in place that govern receptor assembly. $GABA_A$ receptor assembly appears to be a complex process where several factor are involved. Although this process is not yet completely understood a number of studies in the past decade have allowed us to further understand the rules that govern receptor assembly.

With exception of the β1, β3 and γ2S subunits, individual $GABA_A$ receptor subunits are retained in the ER. Moreover, pulse chase experiments demonstrated that α1 and β2 subunits are rapidly degraded when expressed alone (Gorrie et al., 1997) suggesting that these proteins are targeted for degradation from the ER. Importantly, $GABA_A$ receptor subunits have been shown to associate with BiP and Calnexin (Connolly et al., 1996; Gorrie et al., 1997), two chaperone molecules which assist in protein quality control in the ER (Kleizen and Braakman, 2004).

Initial chimeric studies suggested that assembly was dependent on the N-terminus of receptor subunits (Hackam et al., 1997). Using a chimeric approach, four amino acids in the N-terminal domain of the β3 subunit have been identified to mediate functional cell surface expression of this subunit compared to β2 (Taylor et al., 1999). Introduction of these four amino acids into the N-terminus of the β2 subunit is sufficient to enable β2 homomerization and ER exit. These four amino acids are also important for the oligomerization of β subunits with γ subunits but not with α subunits. Interestingly, mutation of these four amino acids within the β3 subunit abolished its ability to form homomers but not its ability to oligomerize with γ2L

suggesting that at least one alternative signal for the assembly of β and γ subunits must exist (Taylor et al., 1999). A conserved domain in the N-terminus of α subunits has also been identified to play a role in the oligomerization of α with β but not γ subunits (Taylor et al., 2000; Bollan et al., 2003). Furthermore, conserved glutamine and arginine residues have been shown to independently play a key role in determining the assembly of α with β subunits (Taylor et al., 2000; Bollan et al., 2003), whereas a conserved arginine in α, β and γ subunits has been shown to be essential for subunit oligomerisation (Hales et al., 2005). Finally, two regions within the N-terminus of the γ2 subunit have been shown to mediate assembly of this subunit with α and β subunits (Klausberger et al., 2000, 2001).

Together the results from the above studies suggest that a number of combined factors play a role in restricting the subunit composition of $GABA_A$ receptors expressed in the brain.

$GABA_A$ receptor localization within the brain

In addition to molecular determination of $GABA_A$ receptor assembly, subunit composition is in part restricted to regional and temporal expression of receptor subunits.

Regional distribution of $GABA_A$ receptors

$GABA_A$ receptors are ubiquitously expressed throughout the brain forming one third of synapses. *In situ* hybridisation and immunohistochemical studies are all in agreement that α1, β2/3, and γ2 are the most abundant $GABA_A$ receptor subunits in the brain (Laurie et al., 1992; Wisden et al., 1992; Pirker et al., 2000). The α1 subunit is found to be present in most brain regions whereas the α4 subunit is restricted to the thalamus, the striatum and the molecular layer of the dentate gyrus and is the least abundant α subunit (Wisden et al., 1992; Pirker et al., 2000). In contrast, α5 is found enriched in the CA1 region of the hippocampus whereas α6 is found almost exclusively in the granular layer of the cerebellum (Laurie et al.,

1992a; Pirker et al., 2000). The α2 and α3 subunits, like α1, are found in most brain regions although at lower levels. Furthermore, an in depth study of of developmental expression revealed that these two subunits are highly expressed neonataly and this expression is reduced in adult brain (Laurie et al., 1992b).

Despite a wide distribution of all three β subunits, β1 is expressed at much lower levels compared to β2 and β3 subunits (Pirker et al., 2000). Interestingly, higher expression of one β subunit in certain brain regions comes at the expense of another. For example, β2 subunits are highly expressed in the thalamus in comparison to β1 and β3 subunits (Wisden et al., 1992; Pirker et al., 2000) whereas the β3 subunit is found to be expressed at high levels in the striatum where very low levels of the β2 subunit are found (Pirker et al., 2000). In addition, β1 and β3 subunits are found at higher concentrations than the β2 subunit in the hippocampus (Wisden et al., 1992) and, in this region, are mainly found in principal neurons whereas β2 subunits are expressed mainly in interneurons (Pirker et al., 2000).

Of the γ subunit isoforms, the γ2 subunit is, by far, the most abundant. However, all three γ subunits are found widely distributed with the γ1 subunit expressed at higher concentrations in the pallidum and substantia nigra whereas the γ3 subunit is slightly more concentrated in the cerebral cortex (Laurie et al., 1992b; Wisden et al., 1992; Pirker et al., 2000). Despite being widely expressed γ1 and γ3 are unable to fully substitute for the γ2 subunit in γ2 knockout mice, which show a lethal phenotype (Essrich et al., 1998).

In contrast to the γ subunits, the δ subunit of $GABA_A$ receptors is specifically concentrated in the granular layer of the cerebellum and in the thalamus where it is thought to be assembled only in receptors that contain α6 and α4 subunits, respectively (Laurie et al., 1992a; Wisden et al., 1992; Pirker et al., 2000).

ε, π and θ are the least abundant $GABA_A$ receptor subunits expressed in the CNS.

The ε subunit has been observed in the hypothalamus, amygdala and brainstem (Davies et al., 1997; Whiting et al., 1997; Moragues et al., 2002). Interestingly, the θ subunit has also been observed in all these regions as well as the substantia nigra and hippocampus (Bonnert et al., 1999, Moragues et al., 2002). The π subunit appears to be expressed mainly in the uterus, although low expression levels have been observed in the hippocampus (Hedblom et al., 1997).

$GABA_A$ receptor subcellular localization

Immunocytochemical and immunogold electron microscopy studies have revealed that $GABA_A$ receptor subunit combinations can be targeted to different subcellular domains. Although the α1-3 and α5 subunits are all found to be expressed in hippocampal and cerebellar cells, studies showed that the α2 subunit is found concentrated in the axon initial segment (AIS) of the majority of cells where it colocalizes with the inhibitory synaptic marker gephyrin (Nusser et al., 1996; Brunig et al., 2002). In contrast, the α1 subunit is expressed throughout the cell and shows both diffuse and clustered staining suggesting both a synaptic and extrasynaptic localisation (Brunig et al., 2002). Interestingly, the α1 subunit cannot be found in the AIS on its own, but only colocalised with the α2 subunit indicating that subunit composition of $GABA_A$ receptors in the AIS consists of one copy of α1 and one of α2 (Brunig et al., 2002).

α3 containing receptors appear to be differentially targeted depending on the cell type where they are expressed. In pyramidal cells, the α3 subunit is found in clusters at postsynaptic sites, whereas in a subset of hippocampal cells, characterised by a round cell body and numerous short dendrites, α3 containing receptors show a diffuse expression pattern across the membrane and are not found at synaptic sites (Brunig et al., 2002). The α5 subunit, on the other hand, is found to be diffuse in all hippocampal cells and never colocalised with gephyrin clusters suggesting that α5 containing receptors are exclusively extrasynaptic (Brunig et al., 2002).

In addition to the α subunit, subcellular localization has also been shown to be determined by the γ and δ subunits. In the cerebellum, immunocytochemical studies on electron microscopic sections demonstrated that synaptic and extrasynaptic $GABA_A$ receptors differed in their subunit composition. Synapses in cerebellar granule neurons were found to be positive for the $GABA_A$ receptor γ2 subunit, whereas the δ subunit was found exclusively in extrasynaptic membranes (Nusser et al., 1998).

Experiments in polarized epithelial cells have suggested a role for the β subunit in subcellular targeting. Heterologous expression of α1β1 complexes resulted in a non-polarized distribution, whereas α1β2 and α1β3 subunit combinations were targeted to the basolateral domain (Connolly et al., 1996). However, to date, there is no evidence for the role of the β subunits in subcellular targeting *in vivo*.

Functional relevance of heterogeneity

Pharmacological studies are beginning to provide us with a better understanding of the functional consequences of $GABA_A$ receptor composition (reviewed in Farrant and Nusser, 2005). There is evidence to suggest that the sensitivity of the receptor to GABA as well as its deactivation rate is defined by the type of α subunit expressed (Lavoie et al., 1997; Bohme et al., 2004; Farrant and Nusser 2005). In addition, the γ2 and δ subunits are important in determining channel opening times as well as being involved in modulating receptor sensitivity to GABA and receptor deactivation (Fisher and Macdonald., 1997; Haas and Macdonald, 1999; Brown et al., 2002; Farrant and Nusser, 2005). Interestingly, in the cerebellum phasic and tonic $GABA_A$ receptor mediated inhibition has been observed and this has been attributed to synaptic and extrasynaptic $GABA_A$ receptors respectively (Brickley et al., 1996, Brickley et al., 1999). Furthermore, in addition to immunocytochemical studies that show γ2 and δ subunits being respectively targeted to synaptic and extrasynaptic sites, electrophysiological studies have confirmed that the γ2 subunit mediates phasic inhibition whereas the δ subunit plays a role in mediating tonic

inhibition (Brickley et al., 1999; Brickley et al., 2001; Stell et al., 2003)

$GABA_A$ receptor associated proteins

Given the selective subcellular distribution of $GABA_A$ receptors it is clear that the trafficking, targeting and clustering of these receptors is a tightly controlled and regulated process. Although the exact molecular mechanisms that govern the subcellular targeting of $GABA_A$ receptors are not yet fully understood, a number of $GABA_A$ receptor associated proteins have been recently implicated in these processes (Table 1).

As outlined above, the $GABA_A$ receptor $\gamma 2$ subunit is not required for the formation of GABA gated chloride channels. However, evidence from neuronal cultures from mice deficient in $\gamma 2$ subunit expression revealed that this subunit is essential for the synaptic localization, clustering and function of $GABA_A$ receptors. A recent study using $\alpha 2/\gamma 2$ chimeras has suggested a role for the TM4 of the $\gamma 2$ subunit to play an important role in the synaptic targeting and clustering of $GABA_A$ receptors although the exact mechanisms on how this is achieved are not yet understood (Alldred et al., 2005). In addition, a yeast two hybrid screen using the $\gamma 2$ subunit as the bait, identified a 17 kDa $GABA_A$ receptor associated protein (GABARAP), which is thought to play a role in the trafficking of $GABA_A$ receptors (Wang et al., 1999). GABARAP binds γ subunits both *in vitro* and *in vivo* and although it is not found at inhibitory synapses it appears to colocalize with $GABA_A$ receptors in intracellular compartments resembling the Golgi and sub-synaptic cisternae (Wang et al., 1999; Kittler et al., 2001). GABARAP shows amino acid sequence similarity to the small microtubule binding proteins GATE-16 and the light chain-3 (LC3) subunit of MAP-1A and 1B and is able to bind microtubules as well as N-ethylmaleimide sensitive factor (NSF) supporting a role for GABARAP in $GABA_A$ receptor intracellular transport (Wang and Olsen, 2000; Kittler et al., 2001). Recent immunochemical and electrophysiological studies have provided evidence to support a functional role for GABARAP in the trafficking of $GABA_A$ receptors to the plasma membrane. Coexpression of GABARAP and

PROTEIN	CELLULAR LOCALISATION	INTERACTION GABAAR	PUTATIVE FUNCTION	REFERENCES
GEPHYRIN	Synapses	No	Clustering/ Scaffold	Craig et al., 1996; Essrich et al., 1998; Levi et al., 2004
DYSTROPHIN	Synapses	No	Stabilization	Knuesel et al., 1999
GABARAP	Golgi	γ2 subunit	Trafficking	Wang et al., 1999; Kittler et al., 2001; Leil et al., 2004; Chen et al., 2005
PRIP-1	Synapses	β3 subuit	Regulation of phosphorylation state/ trafficking	Kanematsu et al 2002 Terunuma et al., 2004
PLIC-1	Intracellular compartments	α & β subunits	Surface number regulation	Bedford et al., 2001
AP2	Clathrin coated pits	β & γ subunits	Endocytosis	Kittler et al., 2000a
HAP1A	Endosomes	β subunits	Endocytic Sorting	Kittler et al., 2004b
BIG-2	Intracellular compartments	β subunits	Trafficking	Charych et al., 2004
GODZ	Golgi	γ subunits	Palmitoylation	Keller et al., 2004
GRIF-1	Intracellular compartments	β2 subunit	Unknown	Beck et al., 2002
gC1qR	Intracellular compartments	β subunits	Unknown	Schaerer et al., 2001

Table 1: $GABA_A$ receptor associated proteins. A number of proteins associated with $GABA_A$ receptor function is described together with their cellular localisation and putative role in the regulation of $GABA_A$ receptor function. Adapted from Arancibia-Carcamo and Moss, 2006.

$GABA_A$ receptors in COS-7 cells results in an increase in the levels of $GABA_A$ receptors expressed at the cell surface (Leil et al., 2004) as well as an increase of GABA mediated currents in oocytes (Chen et al., 2005).

Although GABARAP is not found at synapses, a yeast two hybrid screen and *in vitro* binding studies have identified the synaptic protein GRIP-1 as a binding partner for GABARAP (Kittler et al., 2004a). GRIP-1 (Glutamate Receptor Interacting Protein 1), is a 7 PDZ domain protein that has been shown to play an essential role in AMPA receptor clustering (Dong et al., 1997). GRIP-1 and the 4-PDZ domain splice form GRIP-1c (PDZ 4-7) have also been observed at inhibitory synapses in cultured neurons and in brain slices where it colocalizes with the γ2 subunit of $GABA_A$ receptors (Burette et al., 1999; Dong et al., 1999; Wyszynski et al., 1999; Charych et al., 2004a; Li et al., 2005). The role of GRIP-1 at inhibitory synapses remains unknown, although its ability to interact with GABARAP suggest that it may be involved in the synaptogenesis of inhibitory synapses or in the regulation of $GABA_A$ receptor function (Kittler et al., 2004a).

GABARAP has also been shown to associate with the Phospholipase-C related inactive protein type 1 (PRIP-1) (Kanematsu et al., 2002). Although PRIP-1 does not associate with the γ2 subunit of $GABA_A$ receptors it appears to compete with this for GABARAP binding (Kanematsu et al., 2002). Interestingly PRIP-1 knockout mice showed an impairment in $GABA_A$ receptor modulation by γ2 selective pharmacological agents suggesting that PRIP-1 might play a role in the regulation of $GABA_A$ receptor trafficking by GABARAP ensuring that only mature αβγ receptors are delivered to the plasma membrane (Kanematsu et al., 2002).

Another protein that has been shown to interact with $GABA_A$ receptors is the ubiquitin related protein Plic-1. Plic-1 is a 67 kDa protein with a ubiquitin like N-terminus and a carboxy-terminal ubiquitin associated domain (UBA) (Wu et al., 1999; Kleijnen et al., 2000). Plic proteins are thought to regulate ubiquitin dependent protein degradation in the proteasome by their ability to bind the proteasome and ubiquitin ligases (Kleijnen et al., 2000). Yeast two hybrid screens

and GST affinity purification assays have revealed an interaction between Plic-1 and all α (1-6) and β (1-3) subunits of $GABA_A$ receptors indicating that Plic-1 function may be relevant for the majority of $GABA_A$ receptor subtypes expressed in the brain (Bedford et al., 2001). Immunocytochemical studies found Plic-1 to be mainly expressed intracellularly in the cell body and dendritic axonal processes (Kleijnen et al., 2000; Bedford et al., 2001) although a significant number of $GABA_A$ receptors were found to colocalize with Plic-1 immunostaining beneath the plasma membrane and synaptic sites (Bedford et al., 2001). Interestingly binding to the intracellular loops of α and β subunits is mediated by the UBA domain of Plic-1 and functional studies in HEK293 cells and hippocampal slices revealed that blockade of this interaction results in reduced cell surface expression of $GABA_A$ receptors (Bedford et al., 2001). Consistent with these results, overexpression of Plic-1 in recombinant systems results in an increase in $GABA_A$ receptors expressed at the cell surface without affecting internalization rates (Bedford et al., 2001). The above results suggest that Plic-1 modulates $GABA_A$ receptor cell surface numbers by inhibiting their degradation by the proteasome.

The brefeldin A-inhibited GDP/GTP exchange factor 2 (BIG2) has also been identified in a yeast two-hybrid screen as a $GABA_A$ receptor β subunit interacting protein (Charych et al., 2004b). BIG2 is mainly found concentrated in the trans Golgi network although a small proportion is found in vesicle-like structures along dendrites and near post-synaptic sites (Charych et al., 2004b). BIG2 has previously been implicated in vesicular transport by its ability to catalyse the GDP-GTP exchange on ARF GTPases. Interestingly, coexpression of BIG2 with the $GABA_A$ receptor β3 subunit results in an increase in β3 exit from the ER (Charych et al., 2004b) suggesting that BIG2 is involved in the post-Golgi vesicular trafficking of $GABA_A$ receptors.

Other $GABA_A$ receptor interacting proteins have been identified including GRIF-1 (Beck et al., 2002) and gC1qR (Schaerer et al., 2001). These proteins however are not enriched at synapses and their functional significance remains to be established.

$GABA_A$ receptor clustering

The clustering of $GABA_A$ receptors at postsynaptic sites is thought to be dependent on the presence of the γ2 subunit and the 93 kDa protein gephyrin. Gephyrin has been previously shown to be involved in mediating glycine receptor clustering via a direct interaction with the intracellular domain of the β subunit of these receptors (Pfeiffer et al., 1982; Meyer et al., 1995; Kneussel et al., 1999a). Immunocytochemistry experiments show gephyrin specifically localised at glycinergic synapses in spinal cord, retina and hippocampal neurons. Depletion of gephyrin expression using antisense oligonucleotides inhibits the clustering of glycine receptors (Kirsch et al., 1993) and neuronal cultures from gephyrin knockout mice show diffuse staining of glycine receptors confirming the importance of gephyrin in glycine receptor clustering (Feng et al., 1998).

Interestingly, gephyrin is found clustered at postsynaptic sites in both hippocampal and cortical neurons where glycine receptor expression is relatively low (Craig et al., 1996; Essrich et al., 1998). In these cells, gephyrin colocalises with $GABA_A$ receptors suggesting that gephyrin is a universal inhibitory synaptic marker (Craig et al., 1996; Essrich et al., 1998). Furthermore in cultures from γ2 knockout mice there are no $GABA_A$ receptors accumulated at postsynaptic sites and this loss is parallel to a loss in gephyrin clusters (Essrich et al., 1998). In addition hippocampal neurons treated with gephyrin antisense oligonucleotides, and hippocampal cultures and spinal cord sections from gephyrin knockout mice show a loss of $GABA_A$ receptor α2 and γ2 subunit clusters (Essrich et al., 1998; Kneussel et al., 1999). However, despite these observations it has not so far been possible to show a direct interaction between gephyrin and $GABA_A$ receptors.

Recent observations suggest that the role of gephyrin at gabaergic synapses is to act as a scaffold rather than as a protein required for $GABA_A$ receptor clusterting. The structure of gephyrin together with its ability to bind tubulin suggest that gephyrin can assemble as a lattice and act as a scaffold (Ramming et al., 2000; Schwartz et al., 2001). Gephryin clusters colocalize with all $GABA_A$ receptors independent of

subunit composition however, in hippocampal cultures from gephyrin knockout mice the levels of α2 containing receptors at the cell surface were reduced by 65%, cell surface γ2 containing receptors were reduced by 43% and α1 subunit containing synaptic aggregates were unaffected (Levi et al., 2004). Furthermore, these cultures only showed a 23% reduction in the amplitude of GABA currents compared to wild type neurons (Levi et al., 2004). These results were consistent with previous observations in immature neurons where $GABA_A$ receptor clusters form before gephyrin can be detected at postsynaptic sites (Dumoulin et al., 2000; Danglot et al., 2003). Together these results implicate a role for gephyrin in the clustering of only a subset of $GABA_A$ receptors and suggest a different function at GABAergic synapses. A recent study looked at the role of gephyrin in modulating the cell surface dynamics of $GABA_A$ receptors (Jacob et al., 2005). Using FRAP (Fluorescence Recovery After Photobleaching) and RNAi, they observed that clustered $GABA_A$ receptors were 3 times more motile in cells where gephyrin expression had been impaired compared to control neurons. These results inplicate gephyrin in enhancing the confinement of $GABA_A$ receptors at synaptic sites suggesting that gephyrin, much like PSD-95 at excitatory synapses, acts as a scaffold and aids in the stabilization of previously clustered $GABA_A$ receptors (Jacob et al., 2005).

Another protein found highly concentrated at inhibitory synapses is dystrophin (Knuesel et al., 1999). In brain slices of 2-3 month old rats, dystrophin colocalizes with α2 and γ2 $GABA_A$ receptor subunit clusters found in the soma and dendrites of pyramidal cells as well as α1 and γ2 clusters in the soma and proximal dendrites of Purkinje cells (Knuesel et al., 1999). Interestingly, not all $GABA_A$ receptor clusters are found colocalized with dystrophin indicating that dystrophin is found at a subset of inhibitory synapses (Knuesel et al., 1999). In brain sections from *mdx* mutant mice, which lack dystrophin, $GABA_A$ receptor clusters in pyramidal and Purkinje cells are reduced in size and number whereas gephyrin clusters appear unaltered (Knuesel et al., 1999). Dystrophin is found associated with α-dystroglycan and β-dystroglycan to form the dystrophin associated glycoprotein

complex (DGC). Analysis of both α- and β-dystroglycan localization in cultured hippocampal neurons revealed that, like dystrophin, dystroglycan is found at a subset of GABAergic terminals, although this colocalization was only evident in late stages of development (Levi et al., 2002). In cultured hippocampal neurons from dystroglycan conditional knockout mice, there was no evidence of dystophin accumulation yet $GABA_A$ receptor and gephyrin synaptic accumulation was unaltered (Levi et al., 2002). Together these results indicate that the DGC is not required for $GABA_A$ receptor clustering, but might play a stabilizing role for the receptor at inhibitory synapses.

Modulation of $GABA_A$ receptor function by post-translational modifications

Phosphorylation

Modulation of $GABA_A$ receptor function is thought to be, in part, achieved by the phosphorylation of $GABA_A$ receptor subunits (reviewed in Brandon et al., 2002; Kittler et al., 2003; Song and Messing 2005). Diverse studies on $GABA_A$ receptor phosphorylation have implicated this process in altering channel kinetics, channel open time, rate of desensitization, sensitivity to pharmacological agents as well as channel receptor stability (see below). It has proven difficult to study phosphorylation of $GABA_A$ receptors in their native state which is why the most compelling evidence of direct phosphorylation on $GABA_A$ receptor function comes from the study on recombinant receptors. However, the effects of phosphorylation on $GABA_A$ receptor function are complex and contradictory.

The large intracellular domains of $GABA_A$ receptor β subunits contain a conserved serine residue (S409 in β1, S410 in β2, S408/409 in β3) that can be phosphorylated by a number of kinases *in vitro* including PKA, PKC, PKG and Ca^{2+}/calmodulin dependent protein kinase II (CamKII). The functional effects of $GABA_A$ receptor phosphorylation have been mainly addressed in heterologous expression systems and results appear to be varied and complex. The modulation of channel function

by phosphorylation is dependent on the identity of the β subunit. For example phosphorylation of the β1 subunit by PKA results in a time dependent decrease of GABAergic currents (Moss et al., 1992). In contrast phosphorylation of Ser408 and 409 in the β3 subunit by the same kinase results in an enhanced GABA response whereas the β2 subunit is not a substrate for PKA phosphorylation in expression systems (McDonald et al., 1998).

The effects of PKC phosphorylation on $GABA_A$ receptor function have also been examined in heterologous expression systems. Activation of PKC activity using phorbol esters results in a downregulation of $GABA_A$ receptor function in a manner that is dependent on the phosphorylation of S409 in the β1 subunit, S410 in the β2 subunit. However, the use of PKM, a constitutively active catalytic domain of PKC, produced contradicting results to those obtained with phorbol esters. PKM enhances the the response to GABA in transfected mouse fibroblasts in a manner that is dependent on S409 in the β1 subunit (Lin et al., 1996). The reason for this discrepancy remains unsolved although it has been speculated that different PKC isoforms may produce different responses (Song and Messing, 2005).

In addition, BDNF induced PKC mediated phosphorylation of the β3 subunit results in a transient enhancement of $GABA_A$ receptor function followed by a lasting depression of miniature inhibitory post-synaptic currents (mIPSCs) in both cultured hippocampal and cortical neurons (Jovanovic et al., 2004). This modulation was concurrent with with PKC mediated phosphorylation followed by phosphatase 2A mediated dephosphorylation suggesting that *in vivo* phosphorylation of $GABA_A$ receptor β3 subunits can enhance $GABA_A$ receptor function (Jovanovic et al., 2004).

The γ2 subunit is also a substrate for PKC phosphorylation. *In vitro* studies using GST fusion proteins have shown the γ2 subunit to be phosphorylated by multiple kinases at S327 as well as S343 in γ2L. Interestingly, the results observed above with PKM or PKC activation by phorbol esters have also been shown to be

dependent on the phosphorylation of S327 and S343 in the γ2L subunit. In addition to the enhancement (using PKM) and downregulation (using phorbol esters) of GABAergic function by phosphorylation of the γ2 subunit, the dephosphorylation of the γ2 subunit at S327 in hippocampal neurons by calcineurin has been shown to result in a downregulation of $GABA_A$ receptor function (Wang et al., 2003).

In addition, the γ2 subunit can be phosphorylated by Src kinase on tyrosine residues Y365 and Y367 (Moss et al., 1995). Coexpression of Src kinase with $GABA_A$ receptors in mammalian cells results in increased $GABA_A$ receptor function, an effect that is dependent on tyrosine residues 365/367 in the γ2 subunit (Moss et al., 1995). This effect was also observed in cultured cortical neurons, although only in the presence of tyrosine phosphatase inhbitors suggesting that the γ2 subunit is found basally phosphorylated at these residues (Moss et al., 1995).

Interestingly phosphorylation of $GABA_A$ receptors has also been shown to play a key role in determining the modulation of receptor function by pharmacological agents such as ethanol, barbiturates and benzodiazepines. In oocytes expressing α1β1γ2L subunits activation of PKC by PMA (a potent phorbol ester) inhibited GABA gated chloride currents (Leidenheimer et al., 1993). However, PMA treatment of xenopous oocytes expressing α1β1γ2L GABA receptors showed a two-fold increase on diazepam potentiation of GABAergic function (Leidenheimer et al., 1993). Similarly, PMA treatment also resulted in an enhancement of pentobarbital modulation of GABA response (Leidenheimer et al., 1993). Studies on specific PKC isoenzyme knockout mice have provided further evidence for the role of PKC in the allosteric modulation of $GABA_A$ receptor function. In constrast to the effects of PMA activation of PKC in oocytes expressing $GABA_A$ receptors, PKCε knockout mice have increased sensitivity to both pentobarbital and diazepam (Hodge et al., 1999). However, PKCγ knockout mice show no changes in the sensitivity to pentobarbital or diazepam (Harris et al., 1995). The effects on the modulation of $GABA_A$ receptors by ethanol has also been studied in these mice. Ethanol has been shown to enhance $GABA_A$ receptor function in a number of

neuronal preparations including hippocampal, cortical and spinal cord neurons (Mehta and Ticku, 1988; Soldo et al., 1994; Weiner et al., 1994). Interestingly, this effect is abolished in the presence of PKC inhibitors suggesting that phosphorylation by PKC may play a role in the ethanol modulation of $GABA_A$ receptors. Supporting this evidence, PKCγ knockout mice show reduced sensitivity to ethanol modulation of $GABA_A$ receptors (Bowers et al., 1999). However, PKCε knockout mice show increased sensitivity to ethanol (Olive et al., 2000).

The above data highlights the complexity of $GABA_A$ receptor modulation by phosphorylation. However, studies in knockout mice demonstrate how specific kinase isoforms may play an important role in determining the effects of phosphorylation on $GABA_A$ receptor function.

Palmitoylation

Palmitoylation is a post-translational modification that involves the attachment of the fatty acid palmitate to cysteine residues and has been shown to be involved in the membrane targeting and subcellular trafficking of various proteins including AMPA receptors (Hayashi et al., 2005) and the neuronal scaffold proteins PSD-95 and GRIP (DeSouza et al., 2002; El-Husseini Ael and Bredt, 2002; El-Husseini Ael et al., 2002; Smotrys and Linder, 2004). The intracellular loop of the $GABA_A$ receptor γ2 subunit has been recently identified as a substrate for palmitoylation (Keller et al., 2004; Rathenberg et al., 2004). Using a SOS-recruitment yeast two-hybrid assay, a 14-amino acid cysteine rich domain conserved in the intracellular loops of all three $GABA_A$ receptor γ subunits was found to interact with the palmitoyl transferase GODZ (Golgi-specific DHHC zinc finger domain protein). However, this interaction was not observed in pull down assays, suggesting that any interaction between GODZ and the γ2 subunit is transient (Keller et al., 2004). Similarly GODZ was found to be localized in the Golgi where there was partial overlap with the γ2 subunit in HEK293 cells but not in neurons (Keller et al., 2004). Interestingly, mutating the cysteine residues within the intracellular loop of the γ2 subunit resulted in a loss of $GABA_A$ receptor clusters at the cell surface as

did treatment with the palmitoylation inhibitor 2-BrP (Rathenberg et al., 2004). From these studies it is evident that $GABA_A$ receptor palmitoylation plays a critical role in the trafficking and clustering of $GABA_A$ receptors, although the exact mechanisms by which this is achieved remain unknown.

Ubiquitination

The regulation of $GABA_A$ receptor trafficking by the ubiquitin like protein Plic suggests that $GABA_A$ receptors may also be a direct target for modification by the polypeptide ubiquitin. A recent abstract presented at the Society for Neuroscience annual meeting provided evidence to show that the intracellular loop of the $GABA_A$ receptor β3 subunit contains a number of lysine residues that are substrates for ubiquitination. Mutation of all 12 lysines within the intracellular loop of the β3 subunit resulted in decreased levels of β3 subunit ubiquitination without compromising its ability to form benzodiazepine sensitive heteromeric receptors. Expression of this mutant ($\beta3^{K12R}$) in cultured cortical neurons showed increased cell surface expression of $GABA_A$ receptors than those containing wild type β3 subunits, although the cell surface half life or endocytosis rates of the receptors were not affected. In addition, the rate of insertion at the cell surface of the $\beta3^{K12R}$ subunit was greater than that of the wild type subunit. This study, is the first to show ubiquitination of $GABA_A$ receptors and suggests that ubiquitination of the β3 subunit acts specifically within the secretory pathway to regulate $GABA_A$ receptor insertion at the cell surface (Saliba et al., 2005).

Receptor stability

Altering the number of $GABA_A$ receptors found at synaptic sites is an important mechanism for regulating the strength of synaptic inhibition (Kittler et al., 2000a; Kittler and Moss, 2003; Nusser et al., 1997; Nusser et al., 1998). Therefore a significant amount of research has focused on detailing the mechanisms that control the stability of cell surface receptor populations and their accumulation at inhibitory synapses.

Studies on the lateral mobility of both glycine and AMPA receptors using single particle tracking have revealed that there are significant rates of exchange between synaptic and extrasynaptic pools of these ion channels. Interestingly, synaptic receptors exhibit reduced rates of lateral mobility compared to their extrasynaptic counterparts (Choquet and Triller, 2003; Dahan et al., 2003; Tardin et al., 2003). More recently the lateral movement of $GABA_A$ receptors has been studied in cultured hippocampal neurons. Thomas and colleagues used a novel electrophysiological tagging method to demonstrate that in common with glycine and AMPA receptors, $GABA_A$ receptors are dynamic along the plasma membrane and can readily diffuse in and out of synaptic sites providing a molecular mechanism to regulate the efficacy of synaptic inhibition (Thomas et al., 2005). The ability of $GABA_A$ receptors to diffuse along the plasma membrane was also observed in a different study using FRAP (fluorescence recovery after photobleaching) and pHluorin labeled $GABA_A$ receptors (Jacob et al., 2005). Furthermore, this study showed that extrasynaptic receptors are more mobile than their synaptic counterparts and that this difference in motility is dependent on the postsynaptic protein gephyrin (Jacob et al., 2005). Importantly $GABA_A$ receptors have been recently reported to be inserted and removed from the plasma membrane exclusively at extrasynaptic sites highlighting the importance of lateral diffusion (Bogdanov et al., 2006).

In addition to the ability to undergo lateral diffusion along the plasma membrane, $GABA_A$ receptors have been shown to undergo extensive constitutive endocytosis in both heterologous and neuronal systems. Furthermore, a number of associated proteins have been identified to play a key role in regulating $GABA_A$ receptor internalization and endocytic sorting.

$GABA_A$ receptors can be found localized in clathrin coated pits suggesting that these receptors undergo classical clathrin mediated endocytosis (Connolly et al., 1999a). In addition, $GABA_A$ receptor endocytosis is impaired by conditions that block clathrin mediated endocytosis. In both heterologous recombinant systems and in cultured hippocampal neurons, $GABA_A$ receptor endocytosis is blocked by

hypertonic sucrose (Kittler et al., 2000a), conditions which have been shown to impair clathrin function (Heuser and Anderson, 1989). In addition, blocking dynamin function by overexpression of the catalytically inactive dynamin K44A mutant also results in a reduction of $GABA_A$ receptor internalization (Herring et al., 2003; Kittler et al., 2000a; van Rijnsoever et al., 2005). Interestingly, the infusion of neurons with a peptide that disrupts the association between dynamin and amphiphysin (p4 peptide), and therefore dynamin function, results in an increase in amplitude of inhibitory postsynaptic currents (IPSCs) highlighting the physiological significance of receptor internalization (Kittler et al., 2000). Moreover both β and γ subunits have been shown to bind the clathrin adaptor protein complex AP2, an integral member of the clathrin endocytosis machinery (Kittler et al., 2000a). In addition, $GABA_A$ receptor endocytosis has also been reported to undergo a form of clathrin independent internalization in HEK293 cells although there is not yet any evidence that this mechanism also occurs in neurons (Cinar and Barnes, 2001).

The molecular determinants of $GABA_A$ receptor endocytosis are beginning to be understood. A study using recombinant receptors in HEK293 cells revealed the importance of a dileucine motif present in the β2 subunit, and conserved in the β1 and β3 subunits, of $GABA_A$ receptors for efficient internalization (Herring et al., 2003). In addition, a novel AP2 binding motif, which includes the major sites of serine phosphorylation, has recently been identified in all β subunits of the $GABA_A$ receptor (Kittler et al., 2005). *In vitro* binding studies, revealed that AP2 was able to bind the β3 subunit in its dephosphorylated state but not when phosphorylated by PKC or PKA. Interestingly, activation of dopamine D3 receptors in the nucleus accumbens has been reported to cause a downmodulation of GABAergic currents (Chen et al., 2006). This downregulation of GABAergic currents is inhibited by the p4 peptide or a peptide of the AP2 binding site of the β3 subunit. Furthermore, PKA activation also blocked the dopamine dependent downregulation of GABAergic IPSCs (Chen et al., 2006). Together these results provide direct evidence for the importance of phosphorylation in regulating receptor stability at

the cell surface.

Quantitative measurements of $GABA_A$ receptor endocytosis have revealed that approximately 25% of the total cell surface population of β3 subunit containing receptors are internalized within 30min (Kittler et al., 2004b). Given this high rate of internalization the endocytic fate of internalized receptors is likely to play a critical role in regulating receptor cell surface expression levels. It is emerging that internalized $GABA_A$ receptors can be sorted for two endocytic fates. The majority of internalized receptors constitutively recycle back to the plasma membrane over short time frames (tens of minutes) but over longer time periods (6h) 25% of surface receptors are degraded upon internalization (Kittler et al., 2004b). Furthermore internalized receptors have been shown to localize to a subsynaptic pool associated with postsynaptic proteins such as gephyrin (van Rijnsoever et al., 2005), suggesting high levels of receptor recycling in neurons. Recently huntingtin associated protein-1 (HAP1) has been identified as a $GABA_A$ receptor associated protein (Kittler et al., 2004b). HAP1 binds the intracellular loop of β subunits *in vitro* and *in vivo*. Overexpression of HAP1 in cortical neurons results in a decrease of $GABA_A$ receptor degradation and consequently an increase in $GABA_A$ receptor recycling (Kittler et al., 2004b). Interestingly, HAP1 knockout mice have reduced $GABA_A$ receptor β subunit expression in the hypothalamus (Sheng et al., 2006). Furthermore, insulin has been shown to downregulate HAP1 expression resulting in a reduction of mIPSCs and muscimol binding sites (Sheng et al., 2006). Together, these results identify HAP1 as a regulator of $GABA_A$ receptor endocytic sorting although the exact molecular mechanisms by which this is achieved remain unknown.

Alterations in $GABA_A$ receptor trafficking in neurological disorders

It is evident that there are a number of mechanisms in place to dynamically regulate the cell surface expression of $GABA_A$ receptors and hence synaptic inhibition. Changes in the functional properties of $GABA_A$ receptors are relevant in a number

of neurological disease, however recent evidence has implicated receptor trafficking to play a major role in certain pathologies associated with $GABA_A$ receptors.

Alterations in the GABAergic system have been reported in schizophrenic patients (Blum and Mann, 2002; Benes and Berretta, 2001; Fritschy and Brunig, 2003). A number of studies have reported an increase in muscimol binding sites in the anterior cingulate cortex (Benes et al., 1992), hippocampus (Benes et al., 1996) and prefrontal cortex (Hanada et al., 1997; Benes et al., 1996; Dean et al., 1999). A recent study demonstrated an upregulation of α2 subunit containing $GABA_A$ receptors in the AIS of pyramidal cells (Volk et al., 2002). Interestingly this upregulation of $GABA_A$ receptor expression occurred in parallel to a downregulation of the GABA transporter GAT-1 in the axon terminals of chandelier cells in the prefrontal cortex (Woo et al., 1998).

$GABA_A$ receptor dysfunction has long been implicated in the development of epilepsy and status epilepticus (Jones-Davis and Macdonald, 2003). Genetic studies have provided evidence for mutations in $GABA_A$ receptor subunits in a number of idiopathic epilepsies (reviewed in Macdonald et al., 2003, 2004). However other forms of epilepsy are characterised by profound changes in the subunit composition and expression of $GABA_A$ receptors. Studies in models of temporal lobe epilepsy (induced by the administration of pilocarpine or kainic acid) reported decreased expression of most subunit subtypes (α2, α3, α5, β1, β3, γ2 and δ) in the molecular layer of the dentate gyrus and the pyramidal cell layer of CA1 and CA3 during acute seizures. However, chronically there appears to be an upregulation of most $GABA_A$ receptor subunit subtypes (α1, α2, α4, β1, β3 and γ2) in the molecular layer of the dentate gyrus whereas the pyramidal cell layer of CA1 and CA3 remains with decreased subunit expression (Sperk et al., 1997; Schwarzer et al., 1997; Tsunashima et al., 1997, Bouilleret et al., 2000). Interestingly, the dentate granule cell layer shows a decrease in α1 subunit expression that is accompanied by an increase in α4, β3 and δ subunit expression (Brooks-Kayal et al., 1998;

Schwarzer et al., 1997; Fritschy et al., 1999). Similarly, analysis of $GABA_A$ receptor subunit expression in human hippocampal tissue from patients with temporal lope epilepsy also revealed increased subunit expression and changes in the subcellular distribution of some subunit subtypes (Loup et al., 2000).

Status epilepticus is characterised by acute loss of responsiveness to benzodiazepines suggesting rapid changes in $GABA_A$ receptor function. A recent study by Wasterlain and colleagues has provided evidence to a possible mechanism for the inhibitory loss and pharmacoresistance to benzodiazepines in status epilepticus (Naylor et al., 2005). Following status epilepticus Wasterlain and colleagues observed a decrease in mIPSCs and a relocation of both β and $\gamma 2$ subunits from synapses to the cell interior suggesting that $GABA_A$ receptor endocytosis is upregulated, or there is a reduction in $GABA_A$ receptor recylcing, during status epilepticus (Naylor et al., 2005). In contrast, extrasynaptic $GABA_A$ receptor tonic currents were enhanced following status epilepticus, however changes in the expression of the $GABA_A$ receptor δ subunit have not yet been identified.

Altered $GABA_A$ receptor subunit expression has also been reported in different brain regions in models of Huntington's disease (HD). Following quinolinic acid induced lesions of the striatum there is a fast (within 2hr) and long lasting (>15 months) upregulation of $GABA_A$ receptors in the substantia nigra (Brickell et al., 1999). This upregulation also observed in the globus pallidus of HD patients and is accompanied by a profound reduction in $GABA_A$ receptor expression in the caudate nucleus and putamen (Faull et al., 1993; Kunig et al., 2000). The recently identified role of HAP1 in regulating $GABA_A$ receptor endocytic trafficking may provide new insights to the molecular mechanisms of altered receptor trafficking in HD (Kittler et al., 2004).

Lack of blood flow to the brain (cerebral ischemia) results in neuronal cell death (Lipton, 1999). Recently, ischemia induced neuronal death has been linked to changes in GABAergic transmission (Schwartz-Bloom and Sah, 2001). Following

ischemia, a rapid (within 30min) and long-lasting (several months) reduction in GABA neurotransmission has been observed in *in vivo* and *in vitro* models. In addition, these changes have been associated with a significant loss of $GABA_A$ receptor surface expression within 30min of oxygen deprivation (Alicke and Schwartz-Bloom, 1995). Furthermore, this downregulation of $GABA_A$ receptor expression is coupled with long term changes in the mRNA expression of α and β subunits in hippocampal areas (Li et al., 1993). Interestingly, modulators of $GABA_A$ receptor function (eg. benzodiazepines) and trafficking (eg. insulin) have been shown to exert neuroprotection following ischemia, highlighting the important role played by $GABA_A$ receptors in ischemia induced neuronal death (Schwartz et al., 1995; Schwartz-Bloom and Sah, 2001; Mielke and Wang, 2005).

There is a growing list of CNS pathologies that display altered $GABA_A$ receptor expression, highlighting the significance of further understanding $GABA_A$ receptor trafficking.

The endocytic pathway

Cells have the ability to internalise membrane proteins, lipids, extracellular ligands and soluble molecules by a process known as endocytosis. Although a variety of endocytic pathways have been identified, including phagocytosis, macropinocytosis and caveolae/raft mediated uptake, the most well characterised method of internalisation is clathrin mediated endocytosis (reviewed in (Le Roy and Wrana, 2005; Nichols and Lippincott-Schwartz, 2001)).

Clathrin Mediated Endocytosis

Clathrin mediated endocytosis allows for surface proteins to be transported to specific organelles in coated vesicles (Fig. 1.4). Soluble clathrin consists of both a heavy (190 kDa) and a light (25 kDa) chain (for reviews on clathrin structure and clathrin mediated endocytosis see (Le Roy and Wrana, 2005; Marsh, 2000; Marsh and McMahon, 1999; Takei and Haucke, 2001)). The heavy and light chain complex can form a clathrin triskelion that can in turn assemble into a polygonal lattice, which aids to deform the plasma membrane into a bud or clathrin-coated pit

and subsequently a clathrin coated vesicle (CCV). Recruitment of clathrin to the plasma membrane is achieved by the interaction of clathrin with the adaptor protein (AP) complex AP2. There are four distinct adaptor protein complexes (AP1-4), all of which have been involved in protein trafficking. However, AP2 is the only one that has been shown to play a key role in the formation of CCVs at the plasma membrane (Owen et al., 2004; Robinson, 1987). In contrast, AP1 is associated with golgi-derived CCVs whereas AP3 and AP4 have not yet been fully characterized. AP2 is hetero-tetrameric complex consisting of two large subunits (α and β2) of approximately 100-130 kDa, a medium subunit termed μ2 (50 kDa) and a small 20 kDa subunit termed σ2. The β subunit of AP2 has been shown to directly interact with clathrin heavy chains whereas the μ2 subunit (and potentially the β2 subunit) is able to interact with specific amino acid sequences within the cytoplasmic domains of membrane protein cargo (Collins et al., 2002). In addition to recognition of the signal sequence in protein cargo, AP2 is recruited to the plasma membrane in a manner that is dependent on the binding of the α and μ2 subunit to plasma membrane phosphatidylinositol 4,5 bisphosphate (PIP2) (Jost et al., 1998; Honing et al., 2005).

The α subunit of AP2 has also been shown to interact with a number of accessory proteins which form essential components of the clathrin mediated endocytic machinery. These include AP180/CALM, epsin/eps15, amphiphysin and auxilin (Wakenham et al., 2000). Interestingly all of these proteins have been shown to bind AP2 at the same site as well as clathrin suggesting that these proteins may act at different steps during the life cycle of CCVs (Jarousse and Kelly, 2000).

The conversion of the invaginated clathrin coated pit into a fully formed and detached CCV is achieved by action of the large GTPase dynamin (Jarousse and Kelly, 2000; Praefcke and McMahon, 2004; Sweitzer and Hinshaw, 1998; Takei et al., 1998). Dynamin is recruited to the nascent clathrin coated pit by an interaction between its poly-proline rich domain and SH3 domain in amphiphysin. Upon formation of the CCV hsc70 and auxilin participate in the removal of the clathrin

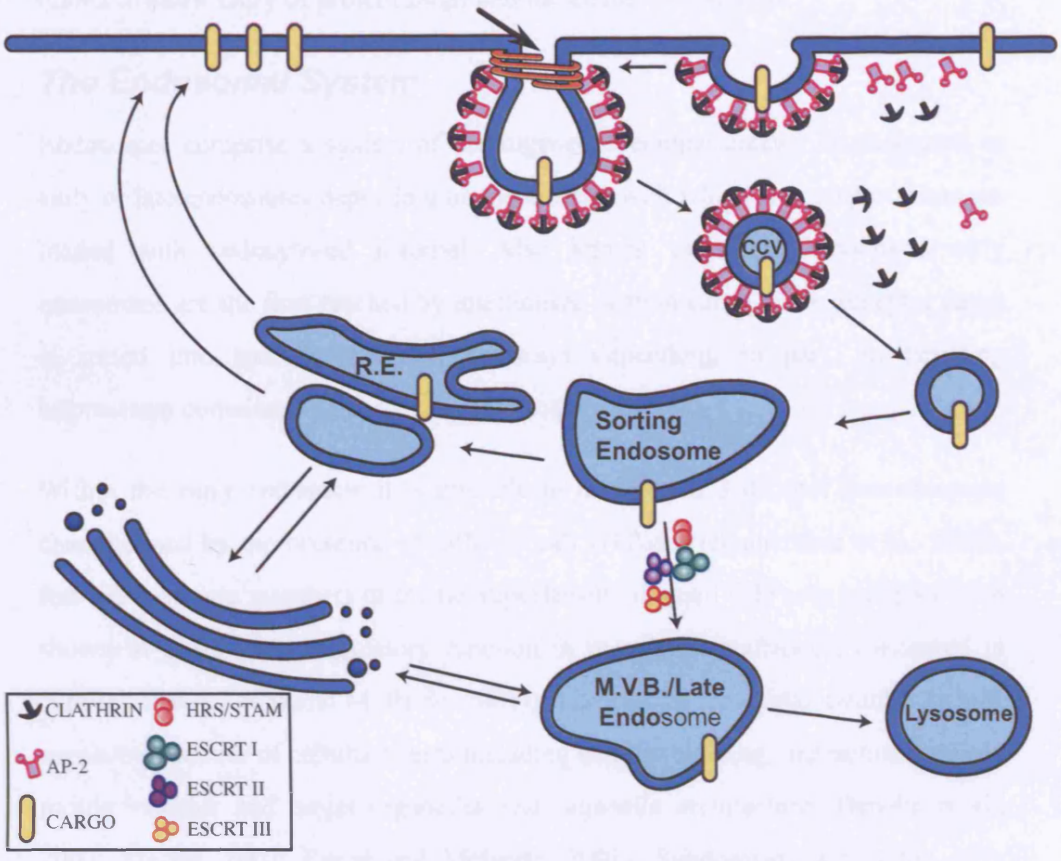


Figure 1.4: The clathrin mediated endocytic pathway. Recruitment of AP-2 and clathrin to the cell membrane, via specific amino acid signals in the intracellular domains of cargo, results in a deformation and invagination of the membrane. Action of the large GTPase dynamin causes the clathrin coated pit to "pinch off" releasing a clathrin coated vesicle (CCV). Upon shedding the clathrin coat, the cargo containing vesicle fuses with the sorting endosome by action of Rab5 and other endosomal proteins such as EEA1. Cargo can then be recycled back to the plasma membrane via the recycling endosome (R.E.) in a manner dependent of Rab4 or Rab11, or can be transported to late endosomes with the aid of Hrs and the ESCRT complexes. From late endosomes most cargo are targeted for degradtion in the lysosome. Adapted from Arancibia-Carcamo et al., 2006.

coat. A number of proteins on the membrane of the early endosome, including rab 5, rabenosyn 5 and early endosomal antigen 1 (EEA1) control vesicle docking and fusion to allow entry of protein cargo into the endosomal system.

The Endosomal System

Endosomes comprise a system of heterogeneous compartements characterised as early or late endosomes depending on the kinetics with which the compartments are loaded with endocytosed material. Also known as sorting endomes, early endosomes are the first reached by internalised protein cargo. Here, receptor cargo is sorted into specific transport pathways depending, in part, on targeting information contained in their amino acid sequences.

Within the early endosome it is possible to distinguish 3 distinct microdomains characterised by the presence of different rab GTPases (Sonnichsen et al., 2000). Rab GTPases are members of the ras superfamily of small GTPases and have been shown to exert a key regulatory function in membrane trafficking (reviewed in (Pfeffer, 2003; Zerial and McBride, 2001)). They act as molecular switches, which regulate a number of cellular events including cellular budding, interaction between motile vesicles and target organelles and organelle architecture (Deneka et al., 2003; Pfeffer, 2003; Zerial and McBride, 2001). Subdomains within the early endosome, containing rab 4 or rab 4 and 11 mature to form recycling endosomes which then are able to deliver protein cargo back to the plasma membrane. A different subdomain of the early endosome, enriched in rab 5, matures to form late endosomes, a process which is characterised by a reduction in the levels of rab 5 and increasing levels of Rab7 (Rink et al., 2005).

Late endosomes eventually mature into lysosomes where their cargo is degraded by lysosomal proteases. In addition to Rab7, several components of the protein machinery thought to be involved in sorting receptor cargo towards the degradative pathway have recently been identified including Hrs/STAM, TGS101, and the ESCRT complexes.

Signals for endocytosis and sorting

A number of sequences for the endocytosis of cell surface receptors and other membrane proteins have been identified (reviewed in (Bonifacino and Traub, 2003)). Most of the signals so far identified have been classified into tyrosine-based signals, dileucine based signals, and motifs that are targets for post-translational modifications.

Classical signals

The sequence motif NPXY was one of the first signals for internalisation to be identified. It was originally identified in the LDL receptor, but later studies found this motif in various cell surface proteins including integrins, and the β-amyloid precursor protein. In addition to the NPXY motif another important family of tyrosine-based signal are the YXXØ signals, where Ø represents any bulky hydrophobic amino-acid (Bonifacino and Traub, 2003). These tyrosine based internalisation signals have been shown to be important for the internalisation of a variety of membrane proteins and receptors including the transferrin receptor.

Tyrosine type sorting signals have not only been implicated in internalisation but have also been found to be important in other sorting events such as lysosomal targeting from endosomes and the trans-golgi network (TGN; (Bonifacino and Traub, 2003; Marks et al., 1995; Williams and Fukuda, 1990). Analysis of the cytosolic domain of the CD3 receptor in T-cells led to the discovery of dileucine-based signals (Letourneur and Klausner, 1992). The [DE]XXXL[LI] motif is found to be highly conserved, and as with the YXXØ motif its function is not restricted to internalization but can also mediate other membrane trafficking events such as lysosomal targeting (Bonifacino and Traub, 2003).

Tyrosine and di-leucine type endocytosis signals have also been implicated in the internalisation of several neurotransmitter receptors including $GABA_A$ receptors and NMDA receptors. Studies using chimeras of Tac and the cytoplasmic tail of the NR2B subunit revealed that the NR2B subunit contained a YXXØ motif that was responsible for internalization (Roche et al., 2001). Furthermore, internalization of

the Tac-NR2B chimera was prevented using dynamin K44A mutant that abolishes the activity of the GTPase dynamin indicating that the internalization of Tac-NR2B is dynamin dependent. NR2A also contains a YXXØ motif within its intracellular tail. However, Lavezzari *et al.* recently showed in a chimeric study that this motif was not involved in the internalization of NR2A, instead mutation of a di-leucine type motif (L1319 and L1320) resulted in a significant reduction of NR2A endocytosis (Lavezzari et al., 2004). In this same study they found that both NR2A and NR2B are able to bind to the μ2 subunit of AP2 although NR2B binds with much higher affinity. Di-leucine type signals have also been implicated in internalisation of $GABA_A$ receptors from the cell surface of HEK293 cells. Herring and colleagues reported that a dileucine motif within the $GABA_A$ receptor β2 subunit is critical for endocytosis and that internalization of $GABA_A$ receptors lacking this motif is dramatically inhibited (Herring et al., 2003).

Atypical signals

In addition, several atypical endocytosis and AP2 adaptor binding motifs have been identified in neurotransmitter receptor intracellular domains. These include basic amino acid rich atypical AP2 binding motifs in both $GABA_A$ receptor (Kittler et al., 2005) and AMPA receptor (Lee et al., 2002) intracellular domains conserved with a previously identified atypical motif in synaptotagmin 1 (Haucke et al., 2000). AMPA receptors also contain other atypical amino acid motifs important for their internalization (Ahmadian et al., 2004; Lin et al., 2000). In addition Scott et al were able to show using tagged NMDA receptor subunits and chimeric constructs that the NR1 subunit also contains a number of endocytosis signals in the membrane proximal domain of its C-terminal tail which defined a novel set of internalization motifs, and which contribute in an additive manner to NMDA receptor endocytosis when expressed with NR2B subunits (Scott et al., 2004).

In addition to non-classical endocytosis motifs, another recently defined atypical signal for internalisation is the covalent modification of lysine residues with the polypeptide ubiquitin, which has also been suggested to act as a membrane sorting

signal. The presence of several endogenous lysines in ubiquitin allows for it to self-ubiquitinate generating poly-ubiquitin chains. Evidence suggests that mono-ubiquitination or small ubiquitin chains act as signals for endocytosis (Hicke, 1999; Pickart, 2001). Evidence of ubiquitin functioning as an internalization signal initially came from studies in yeast where a number of plasma membrane proteins are internalised and targeted for vacuolar degradation by a ubiquitin dependent mechanism (Hicke, 1999). More recently it has also been demonstrated that ubiquitin acts as a signal for endocytosis for a number of mammalian receptors including the EGF receptor (Joazeiro et al., 1999; Levkowitz et al., 1999; Waterman et al., 2002), the epithelial sodium channel (Rotin et al., 2000), E-cadherin (Fujita et al., 2002) and potentially glycine receptors (Buttner et al., 2001). The exact mechanims by which ubiquitination serves as a signal for endocytosis are however still incompletely understood (DiAntonio and Hicke, 2004). Similarly to tyrosine and di-leucine type signals, ubiquitin modification of membrane proteins is also implicated in further trafficking events down the endosomal system through interaction with proteins such as Hrs and the ESCRT complexes, including targeting to multivesicular bodies and late endosomes suggesting that ubiquitination plays an important role in the late stages of the endocytic pathway (Dupre et al., 2001; Rocca et al., 2001).

The role of ubiquitin in the synapse

Ubiquitination has been recently observed as a key process for regulating synaptic function and development. Ubiquitination has been implicated to play key roles in the degradation of proteins at both the level of the lysosome and proteasome. Studies on ubiquitin in neuronal systems have highlighted this process as an important mechanism to control protein stability, activity and localisation at synapses.

Ubiquitin is a highly conserved polypeptide that can be conjugated to lysine chains via its carboxyl-terminal glycine. Ubiquitylation of proteins occurs in three steps: firstly the ubiquitin is activated through the formation of a thiol ester with a cysteine residue of E1 ubiquitin activating enzyme; the ubiquitin polypeptide is

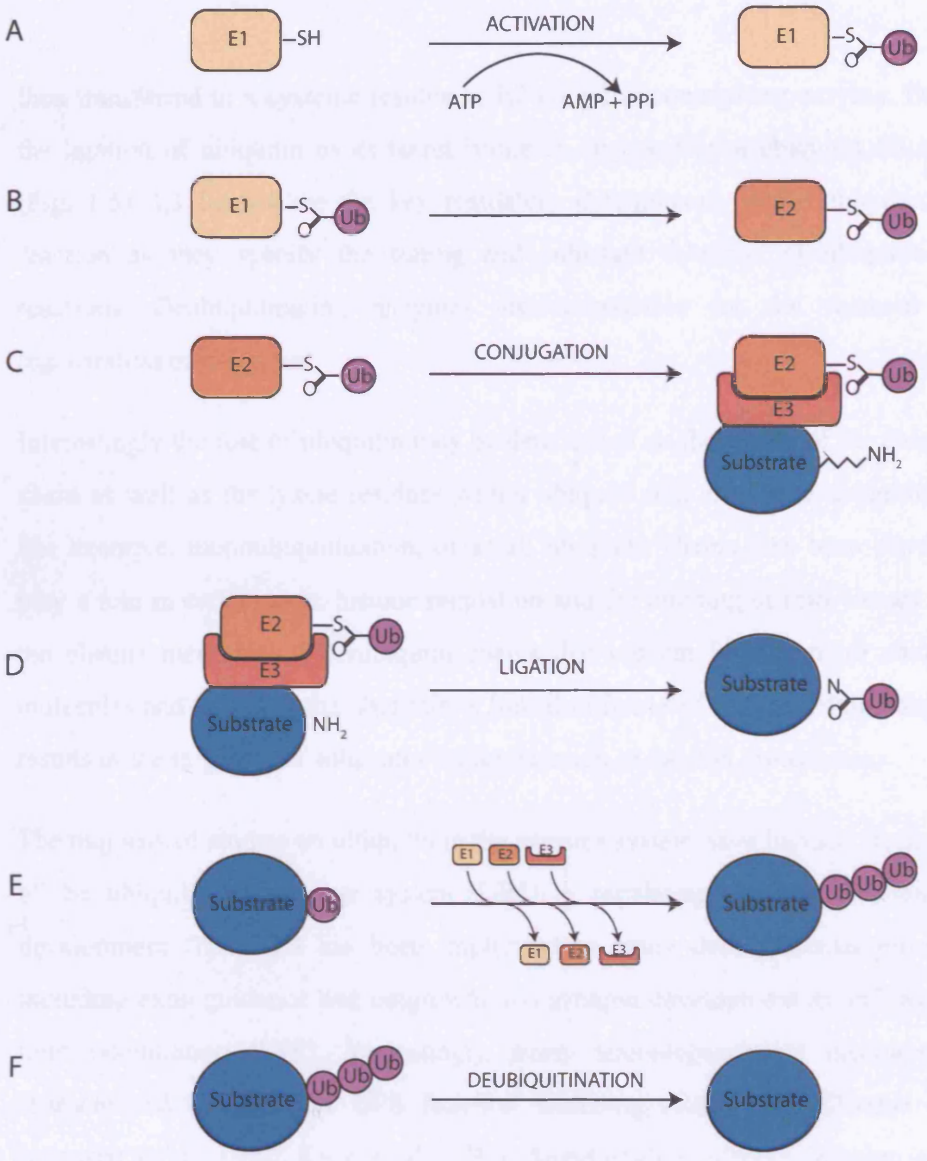


Figure 1.5: Multiple steps in the ubiquitin pathway. (A) Ubiquitin is activated by E1 and ATP hydrolysis. (B) Ubiquitin is then passed on to E2 (ubiquitin carrier enzymes) which is recognised by an E3 ligase bound to the target substrate (C). (D) E3 ligase facilitates the ligation of ubiquitin onto a lysine residue within the target substrate. (E) This process can be repeated to create a polyubiquitin chain by the addition of ubiquitin to lysine residues within the ubiquitin peptide itself. (F) Finally the ubiquitin chain may be removed and the ubiquitin recycled by action of deubiquitinating enzymes (DUBs).

then transferred to a cysteine residue of E2 ubiquitin conjugating enzyme; finally, the ligation of ubiquitin to its target lysine is catalysed by a ubiquitin E3 ligase (Fig. 1.5). E3 ligases are the key regulatory determinants in the ubiquitination reaction as they specify the timing and substrate selection of ubiquitination reactions. Deubiquitinating enzymes are responsible for the removal and regeneration of ubiquitin.

Interestingly the role of ubiquitin may be determined on the length of the ubiquitin chain as well as the lysine residues within ubiqutin that are found ubiquitinated. For example, monoubiquitination, or small ubiquitin chains, has been shown to play a role in endocytosis, histone regulation and the budding of retroviruses from the plasma membrane. Polyubiqutin chains that contain four or more ubiquitin molecules and in which the ubiquitin is linked to Lys48 of the preceding ubiquitin results in the targeting of substrates for degradation in the 26S proteasome.

The majority of studies on ubiquitin in the nervous system have focused on the role of the ubiquitin proteasome system (UPS) in regulating synaptic function and development. The UPS has been implicated in many developmental processes including axon guidance and outgrowth and synapse development as well as long term potentiation (LTP). Interestingly, many neurodegenerative disorders are characterised by aberrant UPS function including Alzheimer's Disease (Van Leeuwen et al., 1998; Keck et al., 2003; Angelman's syndrome (Kishino et al., 1997; Matsuura et al., 1997), Parkinson's Disease (Kitada et al., 1998; Leroy et al., 1998; Shimura et al., 2001), Huntington's Disease (Bence et al., 2001), Ataxia (Wilson et al., 2002; Anderson et al., 2005) and Schizophrenia (MIddleton et al., 2002).

Genetic studies in *Drosophila* and *C. elegans* have provided some of the best evidence in support of UPS function in synapse development. The E3 ubiquitin ligase highwire and the deubiquitinating enzyme fat facets in *Drosophila* have been implicated in regulating synaptic growth and function. Mutation of highwire leads to enlarged synaptic boutons that display abnormal physiology characterised by

reduced mEJPs (miniature junctional potentials) and evoked release despite their normal structural appearance. Similarly, overexpression of fat facets results in similar results suggesting that a balance between ubiquitination and deubiquitination is of crucial importance during synapse development. The highwire *C. elegans* homolog RPM-1 has also been shown to play a role in controlling synaptic growth by acting on both DLK-1 , the *C. elegans* MAPKKK (Nakata et al., 2005), and the receptor tyrosine kinase ALK (anaplastic lymphoma kinase, Liao et al., 2004).

Another E3 ligase that has been recently implicated in neuronal development and synaptic function is the anaphase promoting complex (APC). The APC is a large, multisubunit E3 ligase with critical function in the cell cycle (Harper et al., 2002; Peters et al., 2002). Interestingly, this ligase is highly expressed in neurons in many parts of the CNS including the cerebral cortex, hippocampus and cerebellum suggesting a new role for the APC in the brain (Gieffers et al., 1999). More recently, genetic and RNAi studies in rodents, *Drosophila* and *C. elegans* have identified a role for the APC in regulating axon growth, morphological differentiation of neuronal processes, and synapse size and number (reviewed in Stegmuller and Bonni, 2005).

Neuronal activity causes changes in structure and composition of the PSD in a manner that is inhibited by proteasome inhibition (Ehlers, 2003). Moreover, increases in neuronal activity are mirrored by an enhancement in the ubiquitination of a number of post synaptic proteins including Shank, GKAP, AKAP79/150 and PSD-95 (Colledge et al., 2003; Ehlers, 2003). Interestingly, the ubiquitin dependent degradation of PSD-95 has been shown to play a role in regulating AMPA receptor stability at the plasma membrane (Patrick et al., 2003).

Research into the direct ubiquitination of post synaptic receptors is an active research area. The α1 subunit of glycine receptors has been shown to be ubiquitinated at the plasma membrane, facilitating its internalization and subsequent degradation (Buttner et al., 2001). In addition, $GABA_A$ receptors have

been recently shown to be a target for ubiquitination on their β3 subunit and that this ubiquitination plays a role in determining cell surface expression by regulating insertion rate (Saliba et al., 2005). A recent study by Burbea and colleagues found that the GluR1 subunit of AMPA receptors can be ubiquitinated generating a signal for internalization in *C. elegans* (Burbea et al., 2002). However, the direct ubiquitination of mammalian AMPA receptors has not yet been demonstrated.

Given the importance of receptor trafficking in regulating synaptic strength. This thesis focuses on further undestanding the molecular determinants for regulating the $GABA_A$ receptor endocytic pathway. Furthermore it investigates a possible role for ubiquitin in this pathway and looks at the functional consequences of altering $GABA_A$ receptor endocytic sorting.

2. MATERIALS AND METHODS

Antibodies

9E10 anti-myc antibody was obtained from 9E10 hybridoma cells and used directly as supernatant. Rabbit anti-$GABA_A$ receptor β3 was raised against GST-β3 amino acids 345-408 and purified on an MBP-β3 345-408 column.

Antibody production

Monoclonal 9E10 (anti-myc) and 12CA5 (anti-HA) antibody

12CA5 and 9E10 hybridoma cells were grown in Integra CL350 flasks. Cells were maintained in the cell compartment in DMEM supplemented with 20% FBS. The nutrient compartment contained serum free DMEM. After cells had reached confluency (no more than $2x10^6$/mL) cells were harvested every 3 days. Harvesting involved the removal of 50% of cells and replacement with new media. In addition nutrition media had to be changed with every harvest. Cells removed from each harvest were centrifuged for 2min at 1000rpm. Supernatant containing high concentrations (estimated at 0.5mg/mL) of antibody was filtered through a 0.45μm filter and stored at –20°C.

Purification of 12CA5 anti-HA antibody

Monoclonal anti-HA antibody was purified from supernatant on a protein G column. Briefly, protein G sepharose beads were swollen in 20mM $NaPO_4$ buffer pH 7 and stacked in a disposable column. Supernatant, harvested from 12CA5 hybridoma cells, was loaded twice through the column. After washing with 10 volumes of 20mM NaPO4, bound antibody was eluted with 0.1M Glycine pH 2.5. Eluates were immediately neutralised with 1M Tris pH 8.0. Fractions containing antibody present (determined by optical density (OD) at 280nm) were dialysed at 4°C overnight in PBS pH 7.4. Antibody concentration was calculated by OD reading at 280nm using the following extinction coefficient:

OD_{280} 1.4 = 1mg/mL IgG

The purified antibody was stored at 4°C at a concentration of 0.6mg/ml.

Rabbit Anti-$GABA_A$ receptor β3 subunit antibody

Production of Antisera

Rabbit anti-$GABA_A$ β3 subunit antibody was raised against MBP-β3 345-408. Production of antisera was carried out at Cocalico Biologicals (PA, USA). The procedure consisted of an initial inoculation with 300μg of antigen mixed with complete Freund's adjuvant on day 0. This was followed by a booster injection with 100μg of antigen mixed with incomplete Freund's adjuvant on day 14 and 2 more boosters with 50μg of antigen on days 21 and 49. 10 days after the final boost rabbits were exsanguinated.

Preparation of Affinity Chromatography Coloumn

Rabbit anti $GABA_A$ β3 subunit antibody was purified on a GST-β3 345-408 coupled Affigel (Biorad) column. 4mL of Affigel resin were washed in 10mM $NaHPO_4$ buffer at 4°C. Coupling of GST-β3 to the Affigel resin took place overnight at 4°C on a rotating wheel. The resin was then blocked with 1M ethanolamine pH 8.0 for 30min at 4°C. The column was then washed 5 x 15min at 4°C in different solutions as follows:

Wash 1: 50mL 10mM Tris-HCl pH 7.5

Wash 2: 30mL 100mM Glycine pH 2.5

Wash 3: 50mL 10mM Tris-HCl pH 7.5

Wash 4: 30mL 100mM Triethylamine (TEA) pH 11.5

Wash 5: 50mL 10mM Tris-HCl pH 7.5

The resin was then packed into a 10mL glass column (BioRad).

Antibody purification

Rabbit anti $GABA_A$ β3 subunit antibody was purified from 5mL of serum. Serum was filtered through a 0.45μm filter, diluted 1:5 in dH_2O with protease inhibitors

(100μM PMSF, 20μg/mL leupeptin, 20μg/mL antipain, 5μg/mL pepstatin). Prior to loading the serum the column was washed with 10mL Tris-HCl pH 7.5, 10mL 100mM Glycine pH 2.5, 10mL Tris-HCl pH 7.5, 10mL 100mM TEA pH 11.5 and 10mL Tris-HCl pH 7.5. Serum was loaded onto the column with aid of a peristaltic pump (flow rate approx. 2mL/min) for 4hrs at 4°C. The column was then washed with 10mL Tris-HCl pH 7.5 followed by 30mL Tris-HCl pH 7.5 including 0.5M NaCl. Bound antibody was eluted with 100mM Glycine pH 2.5 in 20 x 1mL fractions. After a wash with 30mL Tris-HCl pH 7.5 a second elution with 100mM TEA pH 11.5 was collected. Protein levels in each fraction were determined by OD reading at 280nm as described above.

Those fractions containing higher concentrations of IgG were pooled and concentrated using an Amicon Ultra centrifugal filter (Millipore). Using this same filter, a buffer exchange was carried out by resuspending the antibody in 2 x 50mL of PBS. The purified antibody was stored at 4°C at a final concentration of 0.5mg/ mL.

Molecular Biology

Constructs

$GABA_A$ receptor cDNA constructs encoding α1, β3 γ2S and γ2L subunits in the expression vector pRK5 have been previously described (McDonald et al., 1998). The subunits were tagged with a 9E10 epitope (EQKLISEEDL) between amino acids 4 and 5 as described previously (Connolly et al., 1996). Chimeric β3/γ2 constructs were made by MJ Lumb as described below. GFP-2FYVE, GFP-Rab 7 and HA-Ub were kind gifts from H Stenmark, F Maxfield, and Y. Fujita, respectively.

Chimeras

The ^{9E10}β3 N-terminus-TM3 and the γ2 intracellular loop and TM4 fragments were obtained by PCR using the oligonucleotides described below. The fragments were then cloned into BamHI/HindIII digested pRK5 in a three-way ligation as shown in Figure 2.1.

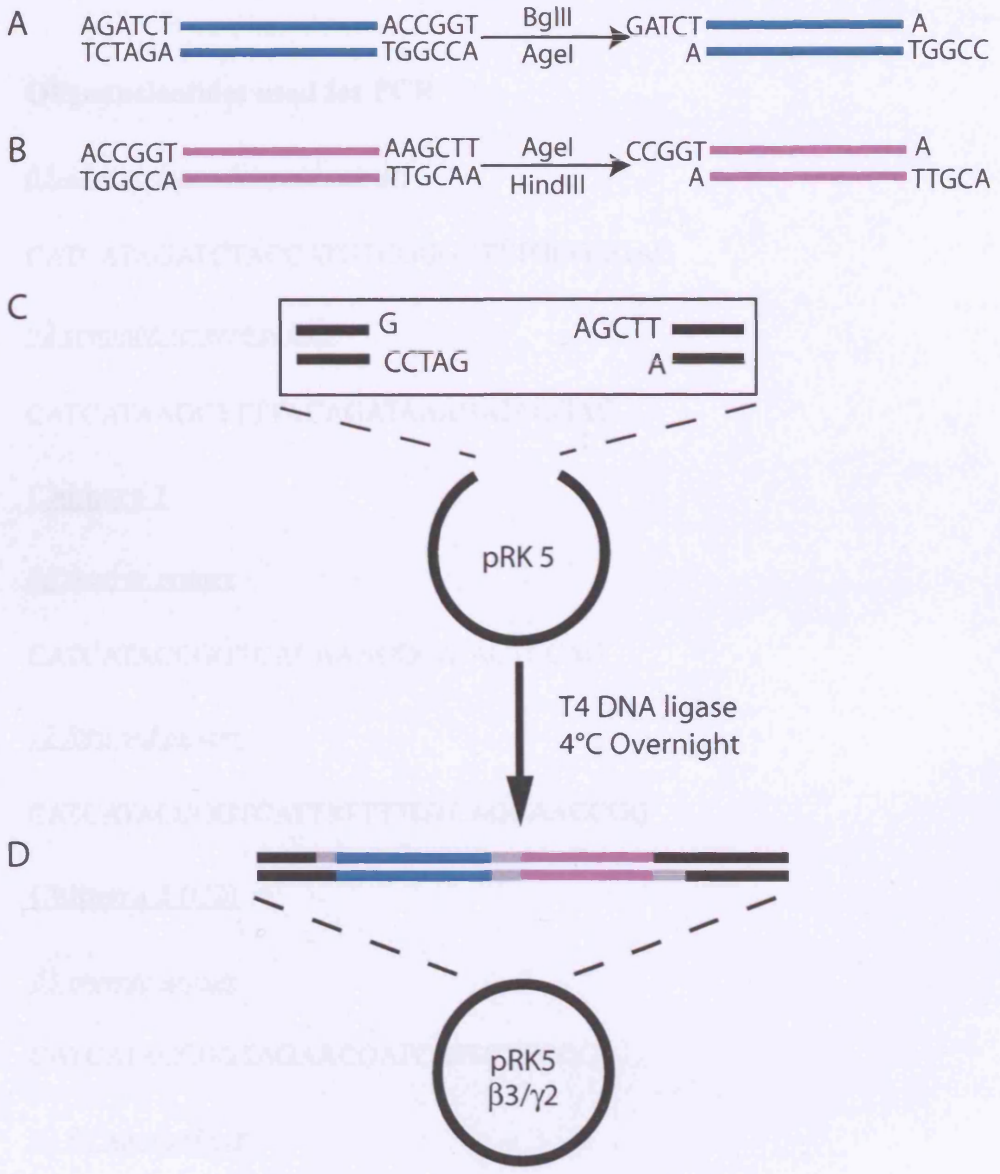


Figure 2.1: Schematic diagram of a 3 way ligation to create β3/γ2 chimeras. PCR products of the β3 (A) and γ2 (B) subunit containing inserted restriction sites are digested using the appropriate enzymes to create 5′ and 3′ overhangs. (C) pRK5 is digested using BamHI andHindIII (sites present in the multiple cloning site (MCS) of the plasmid). All digestion products are incubated at 4°C overnight with T4 ligase allowing for the creation of a β3/γ2 chimera in pRK5 (D).

Oligonucleotides used for PCR

β3-Myc common forward primer

CATCATAGATCTACCATGTGGGGCTTTGCGGGAG

γ2 common reverse primer

CATCATAAGCTTTTACAGATAAAGATAGGAG

Chimera 1

β3 reverse primer

CATCATACCGGTGACAAAGGCATACTCCAG

γ2 forward primer

CATCATACCGGTCATTATTTTGTCAGCAACCGG

Chimera 2 (C2)

β3 reverse primer

CATCATACCGGTAGAACGATCATTCTTGGC

γ2 forward primer

CATCATACCGGTGCCCCTACCATTGATATTC

Chimera 3 (C3)

β3 reverse primer

CATCATACCGGTATCCATCGGTGCTAATAG

γ2 forward primer

CATCATACCGGTCACCTTCAAGAGAGGGATG

Chimera 4 (C4)

β3 reverse primer

CATCATACCGGTTGCTGAATTCCTGGTGTCAC

γ2 forward primer

CATCATACCGGTGCCAGTTTCTTCTGCTGTTTTG

Chimera 5 (C5)

β3 reverse primer

CATCATACCGGTCCGCCCATGCCCTTCCTTG

γ2 forward primer

CATCATACCGGTCGCATTGCCAAAATGGACTC

Growth Media and agar plates

Bacteria were grown in Luria-Bertani (LB) medium at 37°C. Plasmids encoding Ampicilin resistance were grown in 100μg/ml Ampicilin. Plasmids encoding Kanamycin resistance were grown in 30μg/ml Kanamycin.

Transformation of bacteria with plasmid DNA

Plasmid DNA was transformed into One Shot® chemically competent E.coli (Invitrogen) following manufacturer's guidelines. Briefly, one vial of One Shot® chemically competent E.coli was used per transformation. Plasmid DNA was incubated for 5-10min with E.coli cells on ice. Cells were then heat-shocked for 30s at 42°C and immediately returned to ice. Following this bacteria were resuspended in 200μl SOC medium and incubated at 37°C for 1hr prior to plating on LB agar plates. Bacterial colonies were grown on LB agar plates overnight at 37°C.

Maxi-preparation of plasmid DNA

Plasmid DNA was purified from bacterial cultures using Perfectprep® Plasmid Isolation Maxi Kit (Eppendorf). In brief, cells from a 500mL overnight bacterial culture were resuspended in 15mL of Solution 1 and incubated at room temperature for 10min followed by a 5min incubation on ice. Alkaline lysis was carried out on ice for 5min and cells were then neutralized in Solution 3 for 10min on ice. Plasmid DNA was bound to a silica matrix under high chaotropic salt conditions and washed with an alcohol buffer containing low salt. After elution in TE the DNA was concentrated by ethanol precipitation and finally resuspended in molecular biology grade water.

Cell Culture

HEK293 and COS cells

Human Embryonic Kidney (HEK293) or COS cells were grown in NUNC 10cm dishes in 10mL of Dubelco's Modified Eagles Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS). Cells were grown at 37°C in a humidified 5% CO_2 enviroment.

Transient transfection of HEK293 cells

Transient transfections of HEK293 cells were carried using Nucleofector (Amaxa biosystems) technology. Briefly cells seeded 48hrs prior to transfection and at 60-70% confluence were trypsinised for 2 min at 37°C. The trypsin enzymatic reaction was blocked by washing the cells in FBS supplemented DMEM. After centrifugation at 1000rpm for 2 min excess DMEM was removed and the cell pellet was resuspended in 100µl of Nucleofector Solution V per transfection. Cells were transfected with a maximum of 6µg of DNA using Nucleofector program Q-01. Cells were then plated onto poly-L-lysine (10µg/ml) and fibronectin (1µg/ml) coated coverslips at a density of 35000 cells/cm^2 and analysed 24-48 hrs after transfection.

Transient transfection of COS cells

For biochemical experiments, where a large number of cells were needed, transient transfections of COS cells were carried out by electroporation. Two 10cm dishes of cells split the previous day and growing at 50-60% confluency were used per transfection. Cells were trypsinised from the dish and washed once in DMEM. After centrifugation at 1000 rpm for 2min, the cells were washed once in 20ml of Optimem. Cells were centrifuged once more and pellets were resuspended in 0.5ml Optimem per transfection. Cells were transfected by electroporation (0.400V, infinity resistance, 125μF) using a Bio-Rad Gene Electropulser II with a total of 15μg of DNA. Cells were used 40-48hrs after transfection.

Hippocampal Neurons

Embryonic (E18) hippocampal neurons were isolated as previously described (Banker and Goslin, 1998). Briefly, hippocampi were dissected from E18 Sprague-Dawley rats in HEPES buffered Hanks Balanced Salt Solution (HBSS). Cells were dissociated in 0.25% trypsin for 15min at 37°C. Cells were cleared from further trypsin enzymatic activity by three washes in HBSS. Cells were then gently triturated in HBSS using a fire polished glass pasteur pipette followed by trituration in a second fire polished pipette with a diameter 40% smaller than that of the first. Cells were counted using a Neubauer Haemocytometer and cell viability was assessed by erythrosin B exclusion. Dissociated neurons were then plated onto poly-L-lysine (500μg/ml) coated glass coverslips at a density of 14000 cells/cm^2. Neurons were allowed to attach in Minimum Essential Medium (MEM) supplemented with 10% Horse Serum and sodium pyruvate. 2-4 hours after plating the media was replaced with Neurobasal supplemented with 2% B27, 1% glutamine and 33mM glucose.

Cortical neurons

Embryonic (E18) cortical neurons were isolated as previously described (Brandon et al 1999). Briefly, cortices were dissected from E18 Sprague-Dawley rats in HEPES buffered Hanks Balanced Salt Solution (HBSS). Cells were dissociated in 0.25% trypsin for 15min at 37°C. Cells were cleared from further trypsin enzymatic

activity by three washes in HBSS. Cells were then gently triturated in HBSS using a glass 10mL pipette. Cells were counted using a Neubauer Haemocytometer and cell viability was assessed by erythrosin B exclusion. Dissociated neurons were then plated onto poly-L-lysine (50μg/ml) coated 10cm dishes at a density of 40000 cells/cm^2. Neurons were allowed to attach in Minimum Essential Medium (MEM) supplemented with 10% Horse Serum and sodium pyruvate. 2-4 hours after plating the media was replaced with Neurobasal supplemented with 2% B27, 1% glutamine and 33mM glucose.

Transfection of hippocampal cells

Transfections of hippocampal cells were carried using Nucleofector (Amaxa biosystems) technology. Briefly after dissociation of hippocampal cells 1x10^{6}cells were resuspended in 100μl of Rat Neuron Nucleofector Solution per transfection. Cells were transfected with a maximum of 6μg of DNA using Nucleofector program O-03. Cells were then plated onto poly-L-lysine (500μg/ml) coated coverslips at a density of 30000 cells/cm^2.

Imaging

In transfected HEK293 cells

Receptor Cell Surface Labelling

In order to visualise the surface expression of transfected myc-tagged constructs cells were washed in PBS and fixed in 4% paraformaldehyde for 10min at room temperature. Cells were then blocked in PBS containing 0.5% BSA and 10% horse serum. Cells were incubated for 1 hour at room temperature with monoclonal anti-myc antibody diluted in blocking solution, followed by a 1 hr incubation with FITC conjugated anti-mouse. Cells were washed in PBS, mounted with Prolong® Gold Antifade mounting media (Molecular Probes, Invitrogen) and visualised using confocal microscopy.

Receptor Internalisation Assay

Receptor internalization assays were carried out using an antibody feeding protocol as described in Figure 2.2. Live transfected cells were incubated for 30min on ice in HEPES buffer (25mM HEPES, 140mM NaCl, 5.4mM KCl, 1.8mM CaCl, 15mM glucose, pH 7.4) containing 9E10 anti-*myc* antibody. Unbound excess antibody was removed by washing in ice-cold PBS and the cells were returned to conditioned media for a 30min incubation at 37°C. Surface antibody was stripped away using 0.2M acetic acid, 0.5M glycine in PBS prior to fixation as previously described (Carroll et al., 1999). Cells were then fixed in 4% paraformaldehyde for 10min at room temperature and permeabilised in block buffer containing 0.1% Triton. To identify internalised receptors cells were then incubated for 1hr with Texas-Red conjugated goat anti-mouse. Cells were washed in PBS, mounted with Prolong® Gold Antifade mounting media (Molecular Probes, Invitrogen) and visualised using confocal microscopy.

In Cultured Hippocampal Neurons

In order to visualise the surface expression of $GABA_A$ receptors, cultured hippocampal neurons were were washed in PBS and fixed in 4% paraformaldehyde/ 4% sucrose for 10min at room temperature. Cells were then blocked in PBS containing 0.5% BSA and 10% horse serum. Cells were incubated for 1 hour at room temperature with guinea pig anti-γ2 subunit antibody diluted (1:200) in blocking solution. Cells were then washed in PBS and permeabilised for 15min at room temperature in block buffer containing 0.1% Triton. Cells were then incubated for 1hr with anti-VIAAT (to label inhibitory presynaptic terminals) and anti-MAP2 (to visualise dendrites). After washing in PBS cells were incubated for 1hr incubation with Alexa-Fluor 594 conjugated anti-guinea pig, Alexa-Fluor 488 conjugated anti-rabbit and Cy5 conjugated anti-mouse. Cells were washed in PBS, mounted with Prolong® Gold Antifade mounting media (Molecular Probes, Invitrogen) and visualised using confocal microscopy.

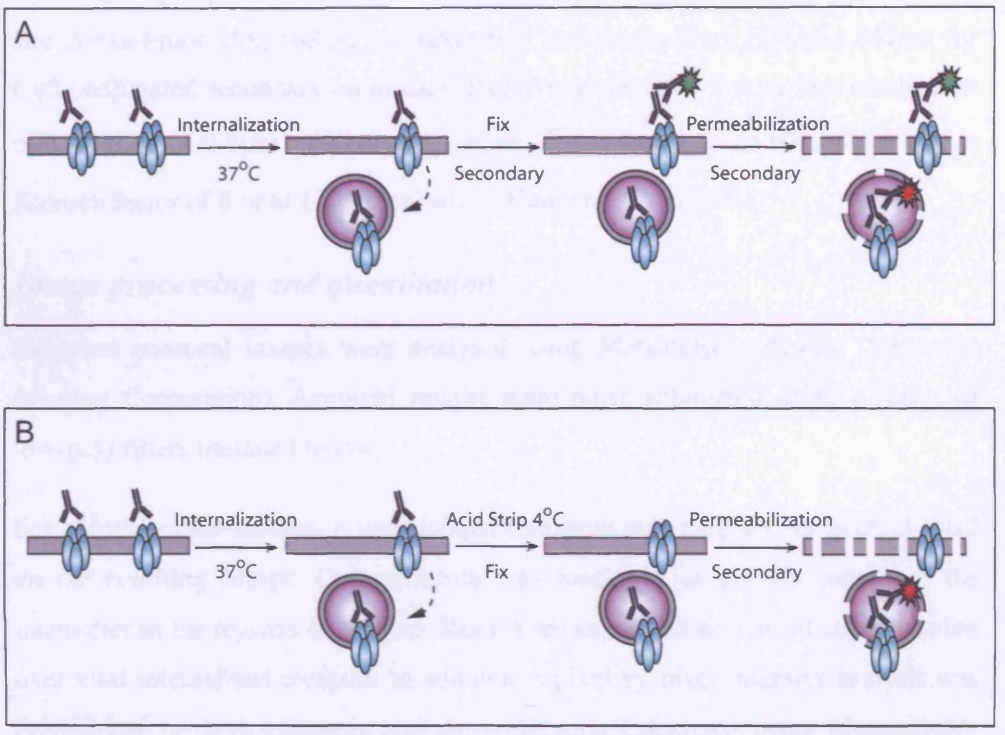


Figure 2.2: Schematic diagram of the antibody feeding technique. (A) Diagram showing the steps involved in the labeling of surface and internalised receptors. Primary antibody to an extracellular epitope is allowed to bind the receptor under conditions that block internalisation. Cells are then returned to conditioned media where receptors are are allowed to internalise. After fixation, addition of a fluorophore conjugated secondary antibody (green) labels all remaining surface receptors. Cells are then permeabilised and incubated with a different fluorophore conjugated secondary antibody (red) to label the pool of internalised receptors. (B) Schematic diagram showing the use of antbody feeding to exclusively label internalised receptors. Binding and internalisation of receptor-antibody complexes are carried out as above. Before fixation, cells are washed in PBS pH 4 to remove any remainnig surface bound primary antibody. Cells are then permeabilised and incubated with a fluorophore conjugated secondary antibody (red) to identify internalised receptors. Adapted from Arancibia-Carcamo et al., 2006

Confocal Microscopy

Cells were viewed using a Zeiss LSM 510 META confocal microscope. All images were digitally captured with LSM software with excitation at 488nm for GFP, FITC and Alexa-Fluor 488, 568nm for Texas-Red and Alexa-Fluor 594 and 647nm for Cy5 conjugated secondary antibodies. Pinholes were set to 1 Airy unit creating an optical slice of 0.8μm. Images were scanned at a speed of 25.60μs/pixel with a Kalman factor of 8 or at 12μs/pixel with a Kalman factor of 16.

Image processing and quantitation

Captured confocal images were analysed using Metamorph software (Universal Imaging Corporation). Acquired images were noise subtracted using a series of low-pass filters (detailed below).

For colocalisation studies, a user-defined threshold was then set for each channel on the resulting image. Colocalization was measured as the integration of the intensities in the regions of overlap. Results are expressed as percent colocalisation over total internalised receptor. In addition, a pixel by pixel intensity analysis was carried out on both channels and the correlation calculated using Metamorph's correlation plot function. The correlation factor ranges from -1, indicating that as pixels brighten in one channel, they become dim in the other channel; to + 1 indicating a linear increase in intensity on both channels.

For cluster identification, images were scaled using a pseudocolor look up table (LUT) on Metamorph. Clusters were defined as an increase in intensity around a central point. Regions were drawn around each cluster and Metamorph software was used to record the size (in μm2) of each cluster.

Low-pass filter

In order to reduce excessive background noise and to correct shading whilst preserving high frequency detail images were subjected to two distinct low pass filters. After an initial shading correction, the image was subjected to a low pass filter with a 3 x 3 kernel. Separately, the image was also filtered using a 7 x 7 low pass filter and the resulting pixel intensities were halved. The final image is a result

from the 7 x 7 filtered image being subtracted from the 3 x 3 filtered image. This process results in an image with enhanced high-frequency spatial detail and low noise levels.

Biochemistry

Biotinylation of cell surface proteins

Cortical cells were washed twice in ice-cold PBS Ca^{2+}/Mg^{2+} (PBS containing 1mM $CaCl_2$ and 0.5mM $MgCl_2$) and incubated for 12min on ice with biotin solution (0.5mg/mL Sulfo-NHS-biotin (PIERCE) in PBS Ca^{2+}/Mg^{2+}). Cells were then washed in PBS Ca^{2+}/Mg^{2+} and quenched for 5min with 50mM glyicne, 1mg/mL BSA in PBS Ca^{2+}/Mg^{2+}. Cells were washed twice in quenching buffer and after a final wash in PBS Ca^{2+}/Mg^{2+} they were scraped in ice-cold RIPA (50mM Tris pH 7.4, 1mM EDTA, 2mM EGTA, 150mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS) containing protease inhibitors and solubilised by rotating 1hr at 4°C. Nuclear and cellular debris was removed by centrifugation at 13000 rpm for 10min at 4°C. 15% of the lysate supernatant was kept for total protein comparison and the rest was precipitated with 50µl of a 50% Ultralink Neutravidin slurry for 2hrs at 4°C. Beads were washed twice in high salt RIPA buffer (containing 500mM NaCl) and once in normal RIPA. Beads were resuspended in 50µl of 3X SDS sample buffer and resolved by SDS-PAGE. Biotinylated surface receptors were then detected by immunoblot analysis using anti-β3 (0.5µg/ml). Results were quantified on a phosphorimager.

Ubiquitin immunoprecipitations

COS cells, transfected with HA-tagged ubiquitin and different chimeras, were washed in ice cold PBS and scraped in lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 2mM EGTA, 1% Triton, 0.1% SDS) containing protease inhibitors. Cells were allowed to solubilse for 1hr at 4°C. Nuclear and cellular debris was removed by centrifugation at 13000 rpm for 10min at 4°C. Chimeras were immunoprecipitated from lysate supernatant using 1µg rabbit anti-myc and 40µl Protein A beads (50% slurry) for 1hr at 4°C. Beads were washed twice in high

salt lysis buffer (lysis buffer containing 500mM NaCl) and once in normal lysis buffer. Beads were resuspended in 50µl of 3X SDS sample buffer and resolved by SDS-PAGE. Immunoprecipitated chimeras were detected by immunoblot analysis using anti-9E10 antibody and ubiquitin levels were detected using anti-HA antibody. Results were detected using ECL chemiluminescence and quantified by densitometry.

SDS-PAGE

3X or 5X SDS PAGE sample buffer was added to all samples prior to loading to a final concentration of 1X SDS PAGE sample buffer (50mM Tris-HCl pH 8.0, 2% SDS, 0.1M DTT, 10% Glycerol and 0.1% Bromophenol Blue). Samples were then loaded in a 1.5mm thick polyacrilamide gel consisting of a low pH (pH 6.8) stacking gel and a higher pH (pH 8.8) resolving gel.

Stacking gels were made as follows (per 10mL):

Protogel (30% acrylamide, National Diagnostics)	1.70mL
dH_2O	6.80mL
1M Tris-HCl pH 6.8	1.25mL
10% SDS	100µl
10 Ammonium persulfate	100µl
TEMED	10µl

Resolving gels were as follows (per 40mL):

dH_2O + Protogel (to required percentage)	29.2mL
1.5M Tris-HCl pH 8.8	10.0mL
10% SDS	400µl
10 Ammonium persulfate	400µl

TEMED	16µl

Gels were run in 1X PAGE buffer (25mM Tris, 250mM glycine, 0.1% SDS).

Transfer of PAGE gels

Resolved proteins were transferred from SDS-PAGE gels onto a Hybond nitrocellulose membrane (Amersham). Briefly, the gel was placed against a pre-wetted nitrocellulose membrane and this was sandwiched between three pieces of 3mm filter paper (Whatman) and a fiber pad on each side. This was then placed in a BioRad transfer apparatus cassette and fitted in a BioRad transfer tank. Transfer was carried out in 1X transfer buffer (25mM Tris, 192mM glycine, 0.037% SDS, and 20% methanol) at 350mA for 3hrs. After transfer the membrane was stained with Ponceau S solution (0.1% Ponceau S, 5% acetic acid) and the positions for the molecular weight markers were indicated in pencil.

Western Blotting

Following transfer the membrane was blocked with 4% milk (Marvel) in PBS-T (PBS containing 0.1% Tween-20) for 1hr. Antibodies were diluted in block buffer to the appropriate concentrations and applied to the membrane in a sealed plastic bag. Antibodies were allowed to bind their epitopes overnight at 4°C on a rotating platform. Excess antibody was washed off with three 10min washes in block buffer. Horseradish Peroxidase (HRP) conjugated secondary antibodies (Jackson Immunoreasearch) diluted in block buffer were incubated with the membrane at room temperature for 1hr. Excess antibody was washed of with three 10min washes in block buffer. The membrane was then rinsed twice in PBS and proteins were detected by application of Super Signal Chemiluminescent substrate (Pierce).

GST-Fusion protein production

BL21 bacteria were transformed with pGEX constructs and grown on LB Agar plates containing Ampicilin. A single colony was grown overnight in 10mL LB containing 50µg/mL Ampicilin and 30µg/mL Chloramphenicol (LB Amp-Chl) at 37°C. This was then grown further in 1L of LB Amp-Chl for 2-3hrs until the culture reached an OD_{600} of 0.5-0.6. Protein expression was then induced by

addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 0.3mM and vigorous shaking at room temperature for 3hrs. Bacterial cultures were centrifuged at 4200rpm for 15min. The bacterial pellet was washed in 10mL of buffer A (50mM Tris-HCl pH 8.0, 25% sucrose, 10mM EDTA), centrifuged again and left overnight at –20°C. The following day the bacterial pellet was allowed to thaw on ice and resuspended in buffer B (10mM Tris-HCl pH 7.4, 1mM EDTA, 1mM DTT, 1mM PMSF) with 1μg/mL of each of the protease inhibitors antipain, pepstatin and leupeptin. Triton-X 100 was added to 1% and the mixture was sonicated at full power for 5 x 15s. Following sonication 25mL of buffer C (20mM HEPES pH 7.6, 100mM KCl, 0.2mM EDTA, 20% Glycerol, 1mM DTT, 1mM PMSF) was added to the mixture as well as 400μl aprotinin and protease inhibitors. After centrifugation at 35000 rpm at 4°C the supernatant was added to 1mL of pre-swollen glutathione-Agarose beads (80mg of beads before swelling) and the mixture was left rotating for 2hrs at 4°C. The beads were then washed 3 x 10min in buffer C. Elution was carried in 1mL elution buffer (buffer C containing 50mM Glutathione, pH 7.5). The mix was centrifuged at 4000rpm for 2 min and the supernatant containing the fusion protein removed. A second elution was carried out to obtain higher yields. Fusion proteins in elution buffer were dialysed overnight in PBS at 4°C using a 10000 MWCO dialysis cassette (Pierce). Fusion proteins were stored at –20°C.

MBP fusion protein production

BL21 bacteria were transformed with pMAL-c2x constructs and grown on LB agar plates containing Ampicilin. A single colony was grown overnight at 37°C in 10mL LB containing 2g/L glucose and100μg/mL Ampicilin. This was then grown further in 1L of LB Amp + glucose for 2-3hrs until the culture reached an OD_{600} of 0.5. Protein expression was then induced by addition of (IPTG) to a final concentration of 0.3mM and vigorous shaking at room temperature for 3hrs. Cells were harvested by centrifugation at 4200rpm for 20min. The bacterial pellet was resuspended in 50mL Column buffer (20mM Tris-HCl pH 7.4, 200mM NaCl, 1mM EDTA) and kept at –20°C overnight. The following day the samples were thawed on ice and sonicated at maximum power for 5 x 15s. Samples were centrifuged at

35000rpm at 4°C for 30min and the supernatant saved and diluted 1:2 in Column buffer. MBP fusion proteins were purified over a 4 cm amylose resin column (NEB). The column was washed with 30mL Column buffer prior to loading the supernatant. The supernatant was run through the column at a flow rate of approximately 2mL/min for 2-3hrs. The column was then washed with 50mL of column buffer and the protein eluted with Column buffer containing 10mM maltose. Fractions containing the highest protein concentrations were pooled, concentrated in an Amicon Ultra centrifugal filter (Millipore) and dialysed overnight in PBS.

Electrophysiology

HEK293 cells were transfected (electroporation) with equimolar mixes of the relevant expression constructs. 24-72 later the transfected cells were recorded in the whole-cell configuration of the patch-clamp technique at room-temperature (holding potential -50 mV). Cells were superfused continously with a Krebs`solution containing (in mM): NaCl 140, KCl 4.7, HEPES 10, glucose 11, $MgCl_2$ 1.2 and $CaCl_2$ 2.5 (adjusted to pH 7.4 with NaOH). Borosilicate pipettes (2-5 MΩ) were filled with (mM) KCl 140, HEPES 10, EGTA 11, $MgCl_2$ 2, $CaCl_2$ 1, Mg^{2+}-ATP 2, and additionally for Leupeptin experiments with 40 μM Leupeptin (adjusted to pH 7.2 with KOH). An Axopatch 200B amplifier and Digidata 1322A (Axon Instruments) software were used for pulse generation, data acquisition (10 kHz), and filtering (2 kHz, 4-pole Bessel filter). Series resistance and membrane capacitance were partially compensated (70-80 %). GABA (5 μM) was rapidly applied at each time point to single cells using a modified U-tube, placed < 150 μm away from the cell of interest (Bogdanov et al., 2006).

mIPSCs were recorded from hippocampal neurons, treated overnight with 400μM leupeptin, using whole-cell patch clamp technique in conjuction with a patch clamp amplifier as previously described (Jovanovic et al., 2004; Kittler et al., 2004b)

Media and reagents

LB medium

Per litre:

10g tryptone

5g yeast extract

10g NaCl

pH 7.0

LB Agar

LB + 15g agar

SOC medium

Per 100mL:

2g tryptone

0.5g yeast extract

0.05g NaCl

20mM Glucose (added after autoclaving)

DMEM

Per 500mL

DMEM (Gibco, Invitrogen)

5mL Penicillin-Streptomycin

50mL FBS

Neuronal Attachment Media

Per 500mL

MEM (Gibco, Invitrogen)

6.6mL Glucose

5mL Sodium Pyruvate

50mL Horse serum

Neuronal Maintenance Media

Per 500mL

Neurosbasal (Gibco, Invitrogen)

10mL B27 supplement (Gibco, Invitrogen)

5mL Penicillin-Streptomycin

6.6mL Glucose

HBSS

Per 500mL

50mL 10X HBSS (Gibco, Invitrogen)

5mL 1M HEPES

445mL dH_2O

10X Running Buffer

0.25M Tris-HCl, pH 7.4

2.5M Glycine

1% SDS

10X Transfer Buffer

0.25M Tris-HCl, pH 7.4

1.92M Glycine

0.37% SDS

PBS

137mM NaCl

2.7mM KCl

10mM Na_2HPO_4

2mM KH_2PO_4

Buffer A

50mM Tris-HCl, pH 8.0

25% sucrose

10mM EDTA

Buffer B

10mM Tris-HCl, pH 7.4

1mM EDTA

1mM DTT

Buffer C

20mM HEPES, pH 7.6

100mM KCl

0.2mM EDTA

1mM DTT

20% Glycerol

3. Mapping the molecular determinants of $GABA_A$ receptor endocytic sorting

Introduction

As outlined in chapter 1, regulating the number of post-synaptic receptors at the cell surface has been described as an important mechanism for synaptic plasticity. A significant amount of research has recently focused on understanding the mechanisms involved in regulating receptor trafficking with particular focus on receptor endocytosis and endocytic sorting.

$GABA_A$ receptors have been shown to be constitutively internalised in a clathrin dependent manner in both heterologous expression systems and cultured neurons by the association of β and γ subunits with the adaptor protein AP2 (Connolly et al., 1999; Kittler et al., 2000). In agreement with these findings, blocking $GABA_A$ receptor endocytosis by disrupting the clathrin endocytic pathway results in an increase in GABAergic currents (Kittler et al., 2000). A recent study has provided a mechanism for regulating the endocytosis of $GABA_A$ receptors. Kittler et al demonstrated how the association of AP2 with the β subunit of $GABA_A$ receptors is regulated in a negative fashion by phosphorylation of a conserved serine residue in the intracellular loop of all β subunits (Kittler et al., 2004b).

In addition the binding of HAP1 to the intracellular loop of the β3 subunit has also been shown to determine the endocytic sorting fate of $GABA_A$ receptors in cultured cortical neurons (Kittler et al., 2004b). Furthermore, downregulation of HAP1 expression results in a reduction of $GABA_A$ receptor β subunit expression and a reduction in mIPSCs (Sheng et al., 2006). Although these results convincingly identify HAP1 as a regulator of $GABA_A$ receptor endocytic sorting the exact molecular mechanisms by which this is achieved remain unknown.

Despite the large number of $GABA_A$ receptor subunits a number of studies have revealed that the majority of benzodiazepine sensitive $GABA_A$ receptor subtypes in the brain consist of 2α2β and 1γ2 subunit. The critical role played by the γ2 subunit in inhibitory synaptic transmission has become more evident over a number of years where it has been shown that this subunit is critical for $GABA_A$ receptor

functional expression and pharmacology. γ2 knockout mice show a lethal phenotype and heterozygous animals show reduced number of inhibitory synapses and this is accompanied by seizures and heightened anxiety like behaviour (Essrich et al., 1998; Crestani et al., 1999). In addition the γ2 subunit has been shown to be responsible for conferring benzodiazepine sensitivity to $GABA_A$ receptors (Pritchett et al., 1989). Furthermore a number of studies looking at the trafficking and subcellular localisation of the γ2 subunit have found this subunit to be essential for $GABA_A$ receptor clustering and synaptic targeting (Essrich et al., 1998; Alldred et al., 2005).

Despite the extensive studies on $GABA_A$ receptor endocytosis very little is known on the role of the γ2 subunit in regulating the endocytic sorting of these proteins. Given the importance of the γ2 subunit it would be interesting to know whether this subunit can confer specific trafficking properties in the endocytic pathway. Initial studies have already suggested that the γ2 subunit may indeed play a role in the endocytic pathway although what specific targeting pathway it follows and whether this can be regulated has not been addressed (Connolly et al., 1999a; Kittler et al., 2000).

In this chapter the role of the γ2 subunit in regulating $GABA_A$ receptor endocytosis and endocytic sorting is addressed by comparing the trafficking properties of recombinantly expressed α1β3 and α1β3γ2L containing receptors in HEK293 cells. The fate of internalised $GABA_A$ receptors composed of α1β3 or α1β3γ2L subunit combinations is compared using antibody feeding coupled with quantitative high resolution immunocytochemistry and confocal microscopy. Furthermore chimeric $GABA_A$ receptor β/γ2 subunits are constructed and used to directly compare and demonstrate the targeting properties of the γ2 subunit intracellular domain and TM4 to that of the β3 subunit. Furthermore, this system is used to identify specific amino acids signals within the γ2 subunit that may regulate the endocytic membrane trafficking of heteromeric $GABA_A$ receptors.

Results

To allow the study of $GABA_A$ receptor endocytosis recombinant $GABA_A$ receptors, where the α1 or the γ2L subunits contained an N-terminus 9E10 (myc) epitope (EQKLISEEDL), were used together with GFP-tagged endosomal markers. Addition of this epitope at this extracellular position is functionally silent with regard to both receptor physiology and pharmacology (Connolly et al., 1996). Both the α1 and γ2L subunits are unable to reach the cell surface when expressed alone and depend on co-assembly with β subunits while unassembled homomeric subunits are retained within the ER (Connolly et al., 1996; Gorrie et al., 1997; Bollan et al., 2003). These rules of assembly, therefore ensure that only multisubunit receptors are visualised at the cell surface when using an anti-9E10 antibody under non-permeabilising conditions.

GFP-tagged endosomal markers were used in this study in order to be able to identify the endosomal structures in which internalised $GABA_A$ receptors were localised. Here, GFP-2FYVE was used as a marker for early endosomes, whereas GFP-Rab7 was used to visualise late endosomes/lysosomes.

GFP-2FYVE consists of two Hrs FYVE finger domains in tandem and has been shown to be specifically localised to early endosomes where it colocalises with EEA1 and transferrin receptor staining (Gillooly et al., 2000). In contrast, no visible colocalisation is observed with Golgi compartment or late endosomal markers (Gillooly et al., 2000). Interestingly, in cells transfected with GFP-2FYVE, endosomes appear to be larger than early endosomes in untransfected cells and expression of GFP-2FYVE has been shown to trap internalised EGF receptors in these endosomes thereby inhibiting EGF receptor transport (but not bulk endosomal membrane transport) to late endosomal compartments (Petiot et al., 2003). Therefore, GFP-2FYVE may also act as a reporter for late endosomal targeting of specific cargo.

Rab7 is a small GTPase that has been shown to be necessary for lysosomal biogenesis by its ability to cycle between late endosomes and lysosomes (Chavrier

et al., 1990; Feng et al., 1995; Gruenberg and Stenmark, 2004). GFP-Rab7 has been used as a tool for following receptor trafficking (Scott et al., 2004) as well as the study of late endosomal and lysosomal biogenesis (Bucci et al., 2000; Rink et al., 2005).

Internalised αβ receptors show reduced levels of colocalisation with GFP-2FYVE compared to αβγ receptors

HEK293 cells were co-transfected with GFP-2FYVE and either $^{9E10}\alpha1\beta3$ or $\alpha1\beta3^{9E10}\gamma2L$. Cell surface receptors were labelled under non-permeabilising conditions in live cells with monoclonal anti-9E10 antibody at 4°C and allowed to internalised for 30min as described previously (Connolly et al., 1999a,b; Kittler et al., 2000). Following this, any remaining surface bound antibody was removed in an acid (pH4) wash, whereas 9E10 labeled internalised receptors were protected from this stripping procedure. Cells were then fixed, and internalised 9E10 labeled receptors were detected using a Texas-Red conjugated anti-mouse secondary antibody. Cells were examined using confocal microscopy as described in the materials and methods section. Colocalisation levels of internalised receptors were then quantified using MetaMorph® software and expressed as a correlation factor or as the percentage of internalised receptor signal overlapping the signal from GFP-2FYVE (see materials and methods).

Both $^{9E10}\alpha1\beta3$ and $\alpha1\beta3^{9E10}\gamma2L$ containing receptors were found to internalise within 30 min as shown by the punctate red staining remaining after surface receptors were stripped using acidic conditions (Fig. 3.1 A-B). Quantitative analysis revealed that 32.1% ± 3 of internalised $^{9E10}\alpha1\beta3$ containing receptors are found in GFP-2FYVE positive endosomes. This low level of colocalisation was also demonstrated by a correlation factor of -0.43 ± 0.04 between β3 and GFP-2FYVE (Fig. 3.1 C-D). In contrast, 65.1% ± 5 of internalised receptors containing α1,β3, and $^{9E10}\gamma2L$ subunits were found in GFP-2FYVE positive endosomes (Fig. 3.1 C). This highly significant ($P<0.01$) increase in colocalisation

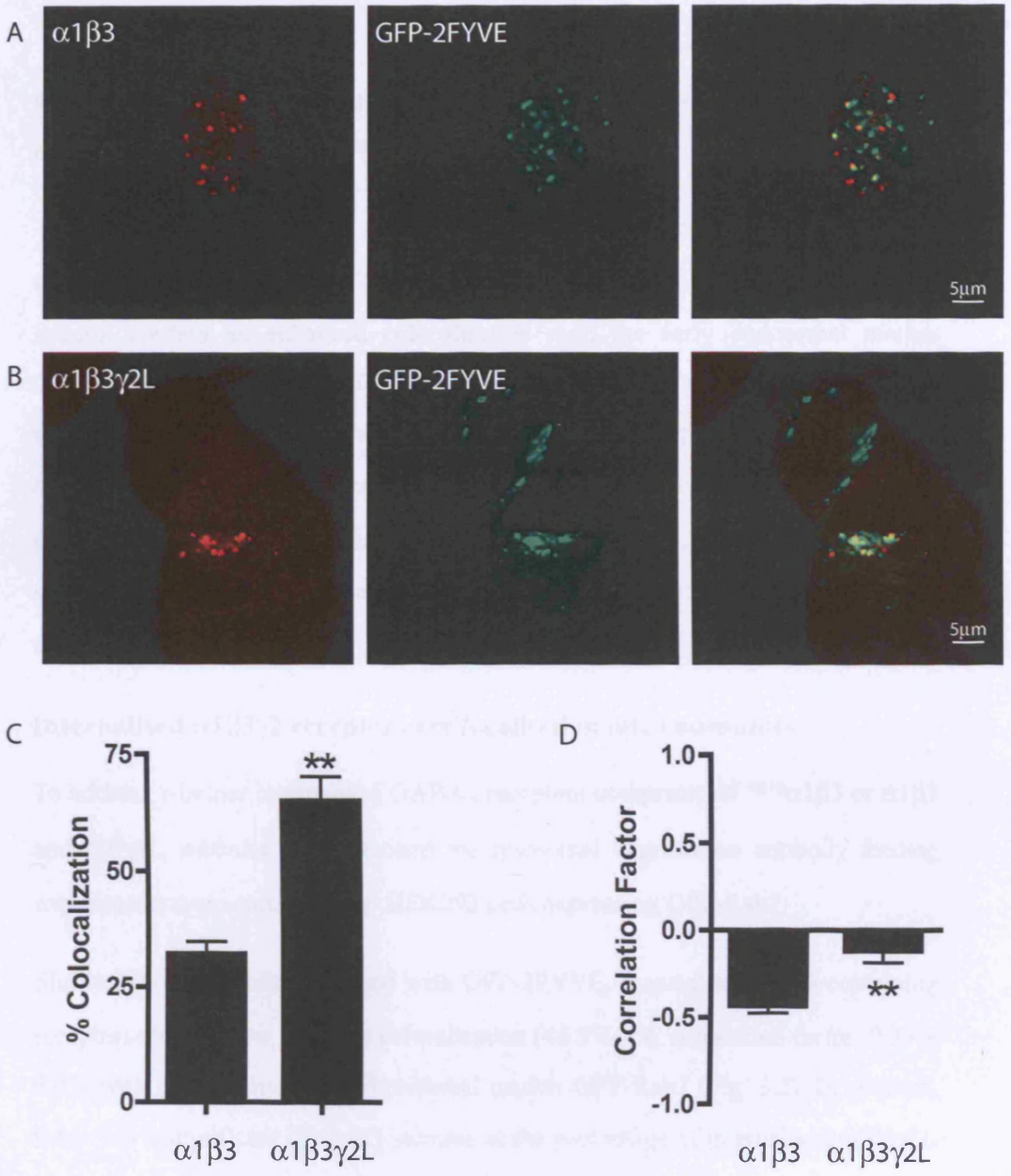


Figure 3.1: The endocytosis of α1β3 and α1β3γ2 receptors in HEK293 cells expressing GFP-2FYVE. Antibody feeding experiments were carried out in HEK293 cells co-expressing α1β3 (A) or α1β3γ2L (B) and the early endosomal marker GFP-2FYVE. (C) Quantitative analysis shows 32.1% ± 3 of internalised α1β3 and 65.1% ± 5 of α1β3γ2L receptors in GFP-2FYVE positive endosomes. This significant ($P<0.01$) enhancement in the colocalisation with GFP-2FYVE endosomes conferred by the presence of the γ2L subunit was observed as a positive shift in the correlation factor (D). (** $P<0.01$, T-test; n=5 (αβ) n=8 (αβγ))

compared to α1β3 containing receptors was also confirmed by an increase in the correlation factor (-0.12 ± 0.07; Fig. 3.1 D).

The above results confirm that $GABA_A$ receptors can undergo constitutive endocytosis in the absence of the γ2 subunit (Connolly et al., 1999). However, this subunit confers an enhanced colocalisation with the early endosomal marker GFP-2FYVE on internalised $GABA_A$ receptors. Given the ability of GFP-2FYVE to accumulate receptors destined to late endosomes in GFP positive endosomes, the above results indicate that $GABA_A$ receptors containing the γ2L subunit may be destined for sorting to a late endosomal/lysosomal pathway. Together, these results suggest that the γ2 subunit may specifically regulate the endocytic fate of $GABA_A$ receptors.

Internalised α1β3γ2 receptors are localised in late endosomes

To address whether internalised $GABA_A$ receptors comprised of ^{9E10}α1β3 or α1β3 and ^{9E10}γ2L subunits were destined for lysosomal degradation antibody feeding experiments were carried out in HEK293 cells expressing GFP-Rab7.

Similarly to the results obtained with GFP-2FYVE, internalised α1β3 containing receptors showed low levels of colocalisation (43.3% ± 4; correlation factor -0.35 ± 0.05) with the late endosomal/lysosomal marker GFP-Rab7 (Fig. 3.2). In contrast, there was a significant ($P<0.01$) increase in the percentage of internalised α1β3γ2L containing receptors found in GFP-Rab7 positive endosomes (67.7 ± 3) as well as a significant increase in the correlation factor (-0.11 ± 0.04) compared to results obtained with α1β3 containing receptors (Fig. 3.2). Together these results suggest that the γ2L subunit confers a specific lysosomal targeting pathway on internalised receptors.

The above results revealed a critical role of the γ2 subunit in targeting internalised $GABA_A$ receptors to late endosomes under basal conditions in HEK293 cells. In keeping with these observations, a role for the γ2 subunit in modulating $GABA_A$

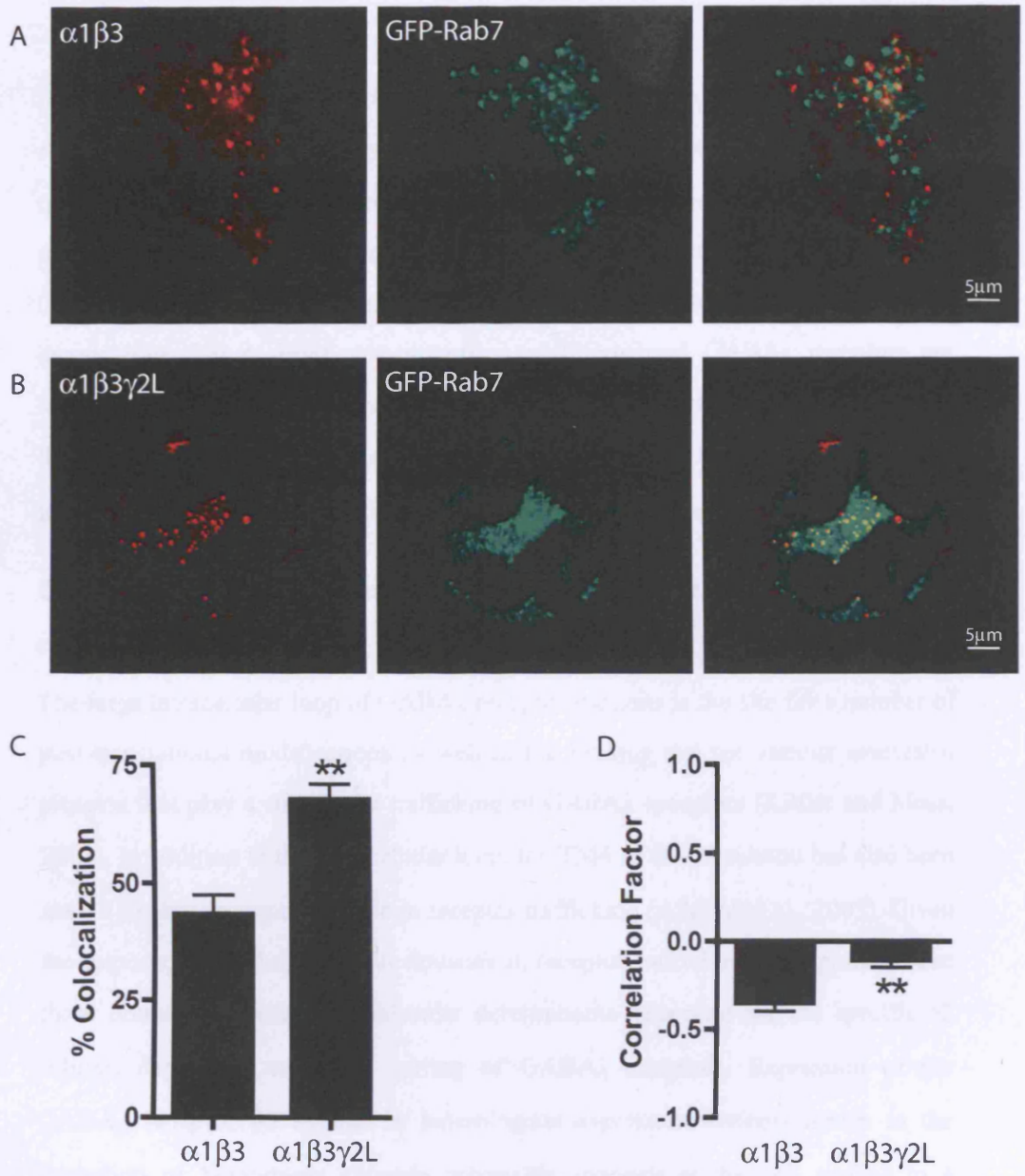


Figure 3.2: The endocytosis of α1β3 and α1β3γ2 receptors in HEK293 cells expressing GFP-Rab7. Antibody feeding experiments were carried out in HEK293 cells co-expressing α1β3 (A) or α1β3γ2L (B) and the late endosomal marker GFP-Rab7. (C, D) Quantitative analysis shows 43.3% ± 4 of internalised α1β3 in GFP-Rab7 positive endosomes and a correlation factor of -0.35 ± 0.05. In contrast, internalised α1β3γ2L receptors and GFP-Rab7 have a correlation factor of -0.11 ± 0.04, where 67.73% of internalised receptors are overlapping GFP-Rab7 endosomes. (** P<0.01, T-test; n=16 (αβ), n=26 (αβγ))

receptor endocytosis has previously been suggested (Connolly et al., 1999). This study demonstrated the ability of α1β3γ2L containing receptors to constitutively internalise and recycle back to the plasma membrane. However, this study did not provide any insights on the proportion of receptors that were recycled or whether $GABA_A$ receptors could be targeted for lysosomal degradation. The above results demonstrate that a significant proportion of internalised $GABA_A$ receptors are targeted for lysosomal degradation. Furthermore, they establish a distinct role for the γ2 subunit in conferring the lysosomal targeting of $GABA_A$ receptors. However, it is not clear what the molecular determinants for this sorting decision may be.

Creating a chimeric system to identify the molecular determinants of endocytic sorting

The large intracellular loop of $GABA_A$ receptor subunits is the site for a number of post-translational modifications as well as the binding site for various associated proteins that play a role in the trafficking of $GABA_A$ receptors (Kittler and Moss, 2003). In addition to the intracellular loop, the TM4 of the γ2 subunit has also been shown to play an important role in receptor trafficking (Alldred et al., 2005). Given the importance of these subunit domains in receptor trafficking, it is possible that these domains contain the molecular determinants important for the specific γ2 subunit dependent endocytic sorting of $GABA_A$ receptors. Expression of the $GABA_A$ receptor β3 subunit in heterologous expression systems results in the formation of homomeric chloride permeable channels at the cell surface in a manner dependent of its N-terminus (Taylor et al., 1999). Therefore, a β3-γ2 chimera consisting of a β3 backbone (N-terminus and TM1-3) with an N-terminal 9E10 tag and the intracellular loop of the γ2 subunit as well as its TM4 was created (Fig. 3.3). This β3-γ2 chimera allowed for a direct comparison between β3 and γ2 subunit dependent endocytic trafficking using a monomeric system and to test whether the intracellular loop and TM4 of the γ2 subunit were important in the endocytic sorting of $GABA_A$ receptors.

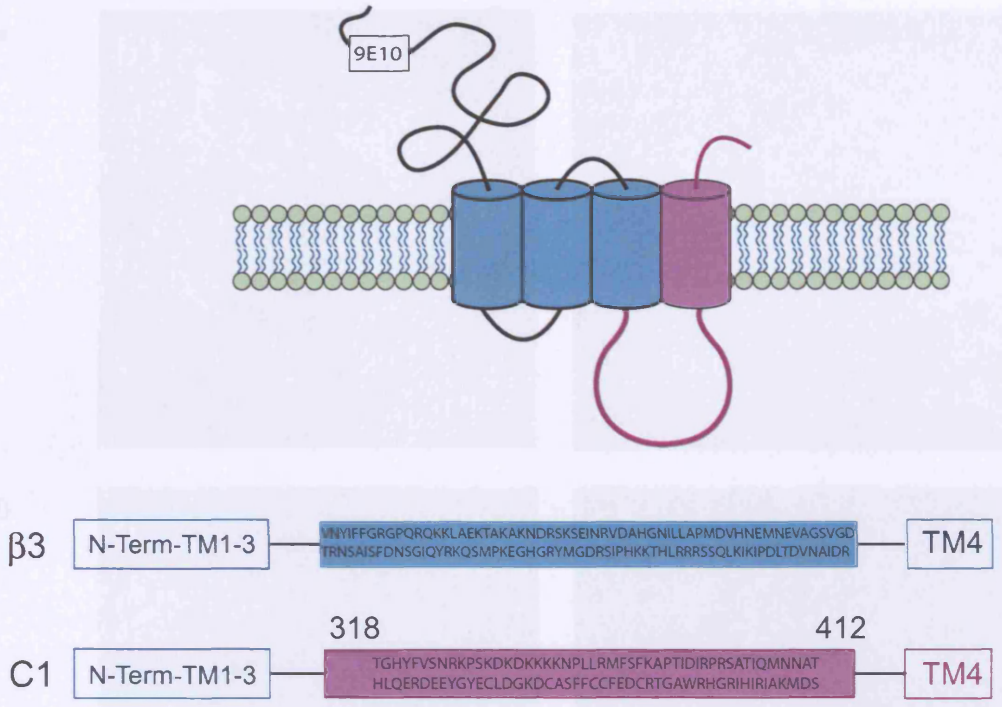


Figure 3.3: Schematic diagram of chimera 1 (C1). C1 consists of a $GABA_A$ receptor β3 subunit backbone (N-terminus and TM1-3) containing an N-terminal 9E10 tag, and the intracellular loop and TM4 of the $GABA_A$ receptor γ2 subunit.

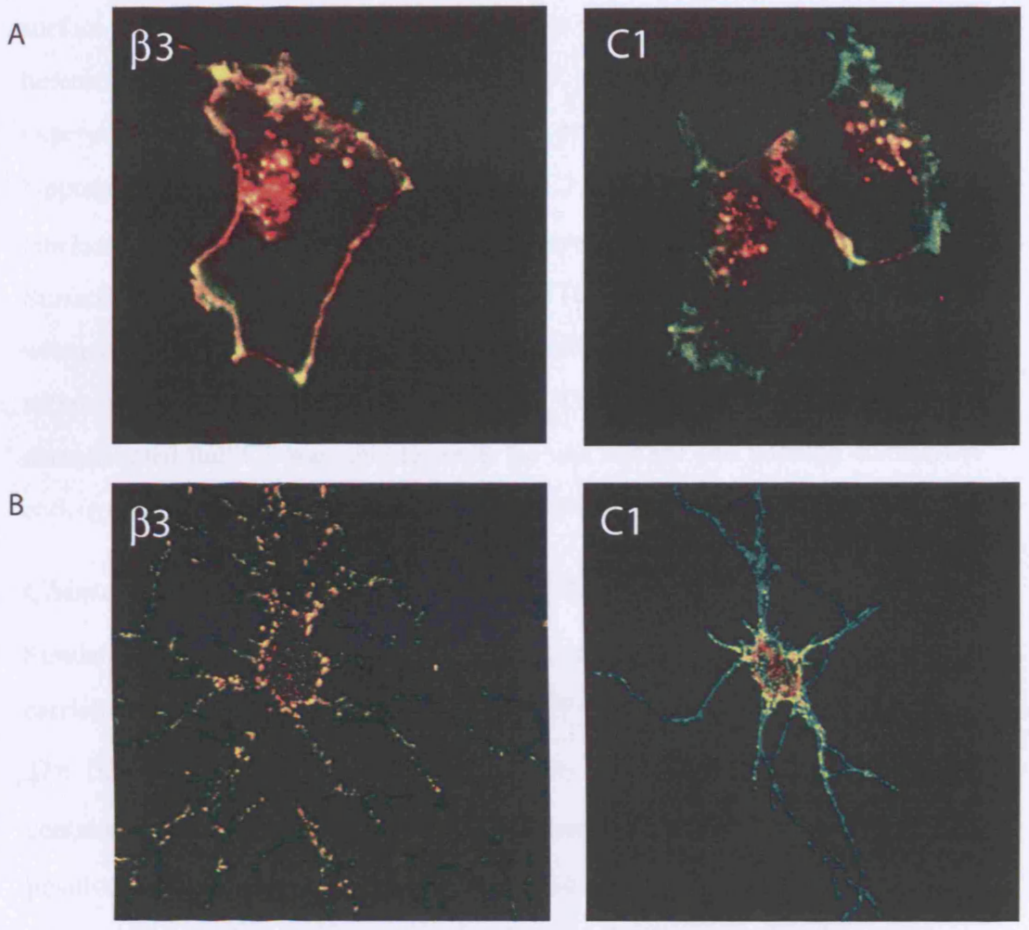


Figure 3.4: Expression of β3 and C1 in both HEK293 cells and cultured hippocampal neurons. Antibody labeling experiments were carried out in HEK293 cells (A) and cutured hippocampal neurons (B) expressing β3 or C1. Cell surface receptors were labelled with anti-9E10 antibody and allowed to internalise for 60 min. Remaining surface receptors were labelled with FITC conjugated anti mouse antibody. Internalised receptors were labelled with Texas Red conjugated antibody.

To test if the β3/γ2 chimera (termed chimera 1 (C1)) was able to reach the cell surface and in addition to undergo constitutive endocytosis in a manner similar to heteromeric $GABA_A$ receptors, a modified version of the antibody feeding experiment, described above, was carried out. In HEK293 cells or cultured hippocampal neurons transfected with β3 or C1, surface expressing receptors were labeled with anti 9E10 antibody and then allowed to internalise at 37°C for 30min. Surface receptors were labeled using a FITC conjugated secondary antibody whereas a Texas-Red conjugated secondary antibody was used to label internalised receptors after permeabilisation of the cells. These antibody feeding experiments demonstrated that C1 was able to reach the cell surface and undergo constitutive endocytosis in both HEK293 cells and hippocampal neurons (Fig. 3.4).

Chimera 1 colocalizes with GFP-2FYVE

Similar to the experiments described above, antibody feeding experiments were carried out in HEK293 cells co-expressing GFP-2FYVE and Chimera 1 (C1) or β3. The β3 subunit was found to constitutively internalize and similar to α1β3 containing receptors 41.3% ± 4 of internalised β3 was found in GFP-2FYVE positive endosomes (Correlation factor -0.34 ± 0.04; Fig. 3.6). In contrast, a substantially higher percentage of internalised C1 was found overlapping GFP-2FYVE positive endosomes (69.6% ± 2). This highly significant ($P<0.01$) increase was confirmed by an increase in the correlation factor (0.09 ± 0.04; Fig. 3.5).

Chimera 1 is targeted to late endosomes

Analogous to the results obtained with GFP-2FYVE, antibody feeding experiments in HEK293 cells expressing GFP-Rab7 and C1 or β3 revealed a significant enhancement in the colocalisation levels of internalised C1 (60.2% ± 2) compared to results obtained with internalised β3 subunit (29.9% ± 4; Fig. 3.6). Together, these results indicate that the intracellular loop and/or the TM4 of the γ2 subunit contains a molecular signal to confer the lysosomal targeting of $GABA_A$ receptors.

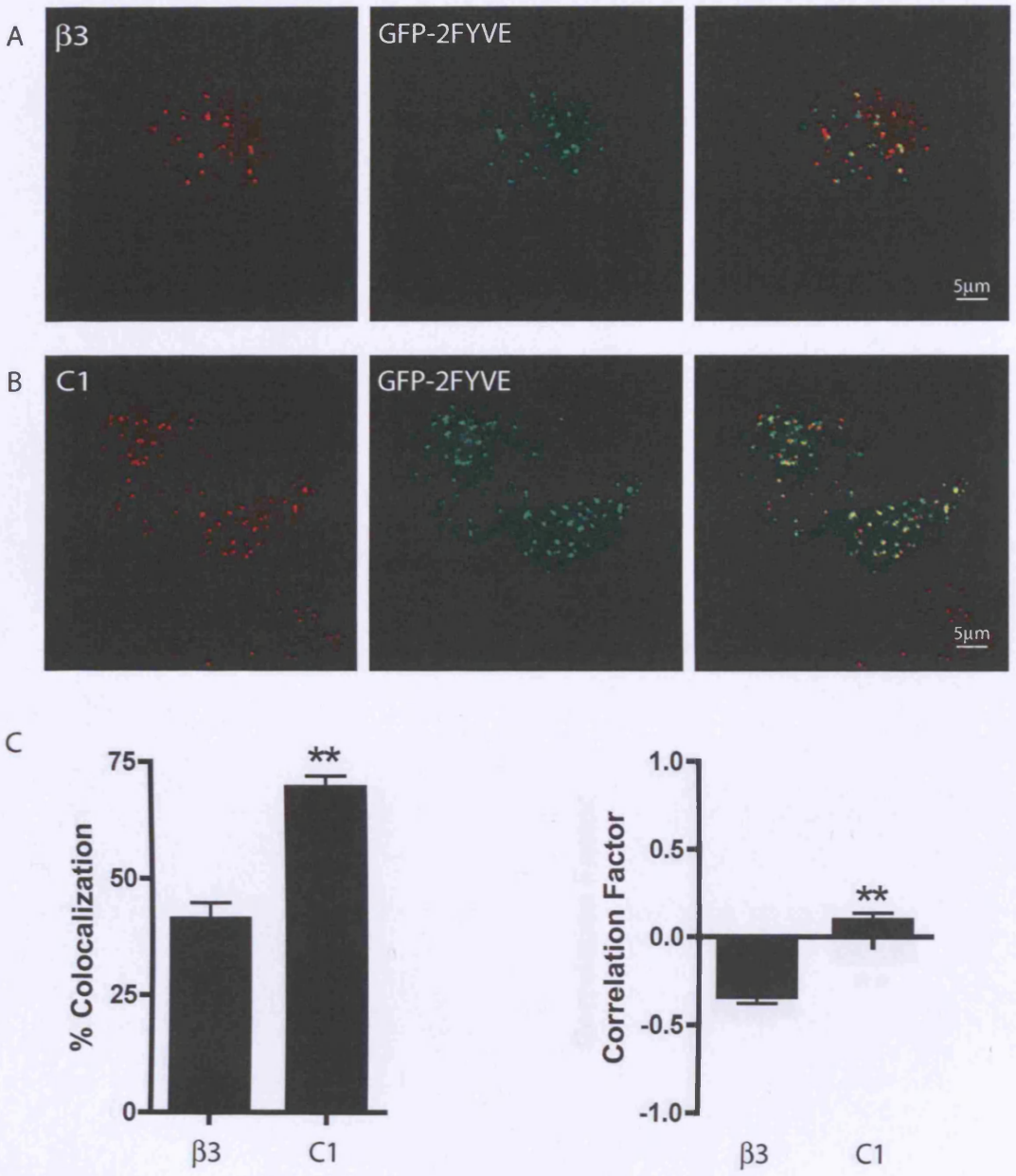


Figure 3.5: The endocytosis of β3 and C1 in HEK293 cells expressing GFP-2FYVE. Antibody labelling experiments were carried out in HEK293 cells co-expressing β3 (A) or C1 (B) and the early endosomal marker GFP-2FYVE. (C, D) Quantitative analysis shows 41.3% ± 4 of internalised β3 colocalised with GFP-2FYVE positive endosomes and a exhibits a correlation factor of -0.3389. In contrast, 69.6% ± 2 of internalised C1 is found in GFP-2FYVE positive endosomes and this is reflected by a significant increase (+ 0.43) in the correlation factor compared to β3. (** P<0.01, T-test; n=29 (β3), n=34 (C1))

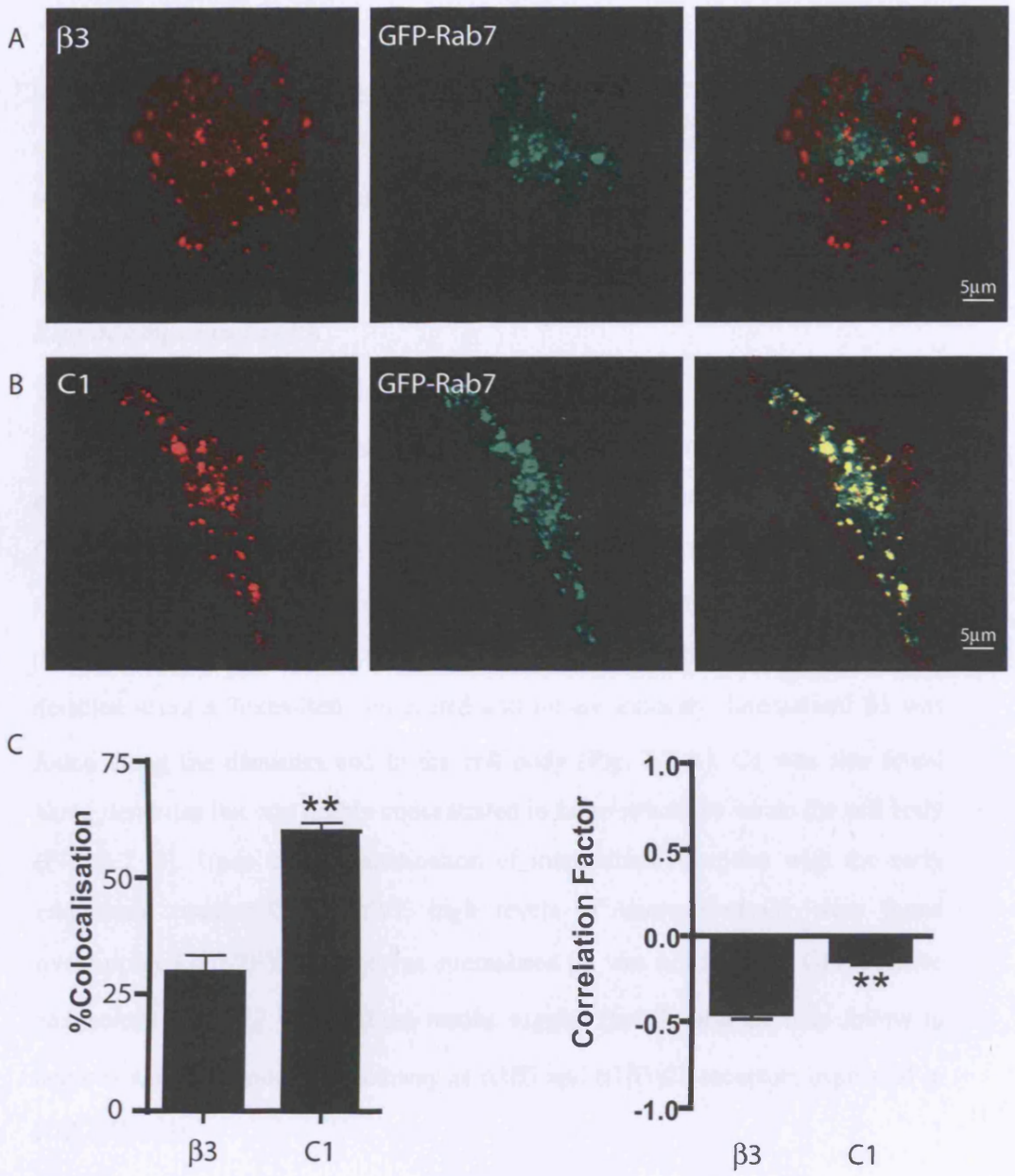


Figure 3.6: The endocytosis of β3 and C1 in HEK293 cells expressing GFP-Rab7. Antibody labelling experiments were carried out in HEK293 cells co-expressing β3 (A) or C1 (B) and the late endosomal marker GFP-Rab7. (C, D) Quantitative analysis shows 29.9% ± 4 of internalised β3 colocalised with GFP-Rab7 positive endosomes and the two channels exhibit a correlation factor of -0.45 ± 0.03. In contrast, 60.2% ± 2 of internalised C1 is found in GFP-2FYVE positive endosomes and this is reflected by a significant increase (+ 0.31) in the correlation factor compared to β3. (** P<0.01, T-test; n=12 (β3), n=27 (C1))

Furthermore, these results establish a single subunit chimeric system as an appropriate method for identifying the molecular determinants of the endocytic sorting of γ2 subunit containing $GABA_A$ receptors.

Chimera 1 co-localises with GFP-2FYVE when co-expressed in hippocampal neurons.

To examine the relevance of these recombinant experiments the targeting of β3 and C1 was investigated in cultured hippocampal neurons. Antibody feeding experiments were carried out in hippocampal cells co-expressing GFP-2FYVE and C1 or β3. Antibody bound β3 and C1 were allowed to internalise for 30min at 37° C. Antibody bound surface receptors were detected using Cy-5 conjugated anti mouse antibody. Cells were then permeabilised and internalised receptors were detected using a Texas-Red conjugated anti mouse antibody. Internalised β3 was found along the dendrites and in the cell body (Fig. 3.7 A). C1 was also found along dendrites but was highly concentrated in large structures inside the cell body (Fig. 3.7 B). Upon closer examination of internalised receptors with the early endosomal marker GFP-2FYVE high levels of internalised C1 were found overlapping GFP-2FYVE, whereas internalised β3 was not found in GFP positive endosomes (Fig. 3.7 C-D). These results suggest that β3 and C1 may follow in neurons a similar endocytic pathway as α1β3 and α1β3γ2L receptors expressed in HEK293 cells.

A tyrosine motif is not necessary for $GABA_A$ receptor lysosomal targeting

The above experiments with β3 and C1 demonstrate that the intracellular loop and TM4 of the γ2 subunit is sufficient to determine the endocytic sorting of $GABA_A$ receptors. A number of molecular signals have been shown to play role in the endocytic sorting of membrane proteins, the most predominant of them being the tyrosine motif YXXØ, where Ø represents any bulky hydrophobic amino acid (see intro; Bonifacino and Traub, 2003). Analysis of the protein sequence of the γ2

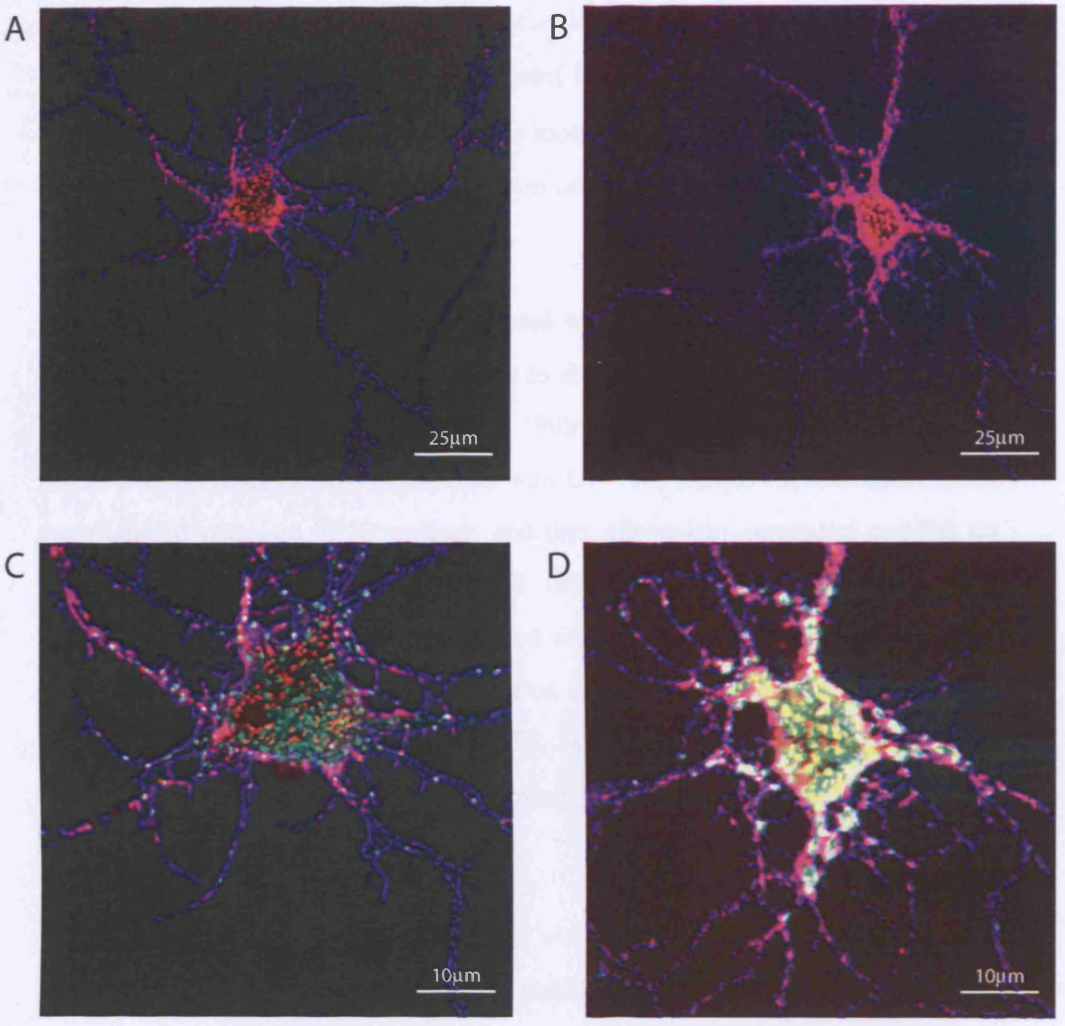


Figure 3.7: Internalisation of β3 and C1 in hippocampal neurons expressing GFP-2FYVE. Antibody labeling experiments were carried out in mature hippocampal neurons transfected with GFP-2FYVE and β3 or C1. Cell surface receptors were labelled with anti 9E10 antibody and allowed to internalise for 60 min. Remaining surface receptors were labelled with Cy5 conjugated anti mouse antibody. Internalised receptors were labelled with Texas Red conjugated antibody. Internalised β3 is found along dendrites and in small aggregates in the cell body (A) where it does not colocalise with GFP-2FYVE (C). Internalised C1 is found in large aggregates concentrated in the cell body (B) where it is highly colocalised with GFP-2FYVE (D).

intracellular loop revealed the presence of a classical tyrosine based internalization and endocytic sorting signal which is absent in the intracellular loops of α and β subunits. In order to address whether this motif could play a role in the endocytic sorting of $GABA_A$ receptors, a mutated version of C1 was used together with the early endosomal marker GFP-2FYVE.

Y^{367} in C1 was mutated using site directed mutagenesis to an alanine residue, a mutation that has been previously shown to abolish tyrosine based signal function in other receptors (Williams and Fukuda, 1990; Haucke and de Camilli, 1999; Li et al., 2000). In HEK293 cells transfected with $C1^{Y367A}$, surface expressing receptors were labeled with anti 9E10 antibody and then allowed to internalise at 37°C for 30min. Surface receptors were labeled using a FITC conjugated secondary antibody whereas a Texas-Red conjugated secondary antibody was used to label internalised receptors after permeabilisation of the cells. These antibody feeding experiments demonstrated that $C1^{Y367A}$ was able to undergo constitutive endocytosis suggesting that this tyrosine motif is not necessary for the internalisation of the γ2 subunit (Fig. 3.8).

Similar antibody feeding experiments were carried out to investigate the accumulation of $C1^{Y367A}$ in GFP-2FYVE positive endosomes. Quantitative analysis revealed that 73.9 ± 4% of internalised $C1^{Y367A}$ is found in GFP-2FYVE positive endosomes expressed in HEK293 cells (Fig. 3.9). This high degree of colocalisation was confirmed by the positive correlation factor (0.11 ± 0.06). These results were highly significant ($P<0.01$) compared to those obtained with the β3 subunit, but did not show a significant change compared to C1. These results suggest that Y367 does not play a significant role in determining the endocytic sorting fate of γ2 containing $GABA_A$ receptors under basal conditions at least in HEK293 cells.

Chimeras 2-5 do not accumulate in GFP-2FYVE endosomes

The above results suggest that an alternative late endosomal/lysosomal targeting signal must exist within the intracellular loop or TM4 of the γ2 subunit. In order to

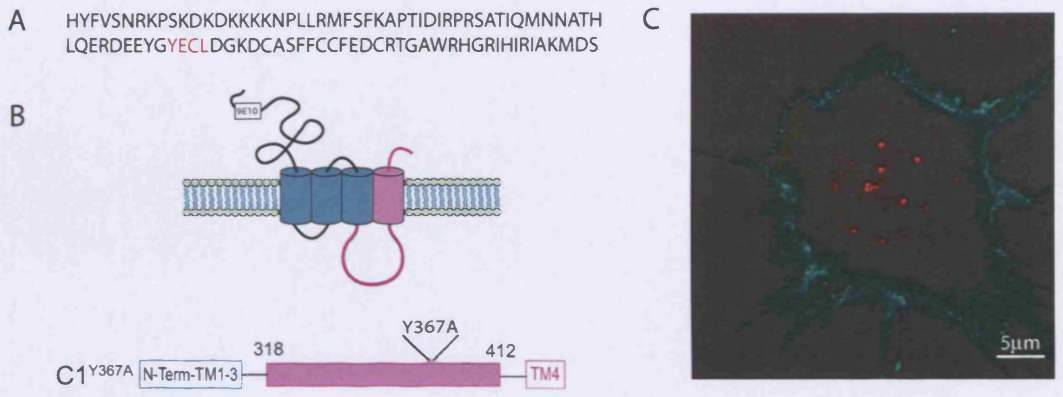


Figure 3.8: Expression of C1^{Y367A} in HEK293 cells. (A) Sequence of the intracellular loop of the γ2S subunit. A classic tyrosine (YXXØ) motif is highlighted in red. (B) Schematic diagram of C1^{Y367A}. (C) C1^{Y367A} was able to reach the cell surface (green) and undergo constitutive endocytosis (red) as demonstrated by antibody feeding experiments.

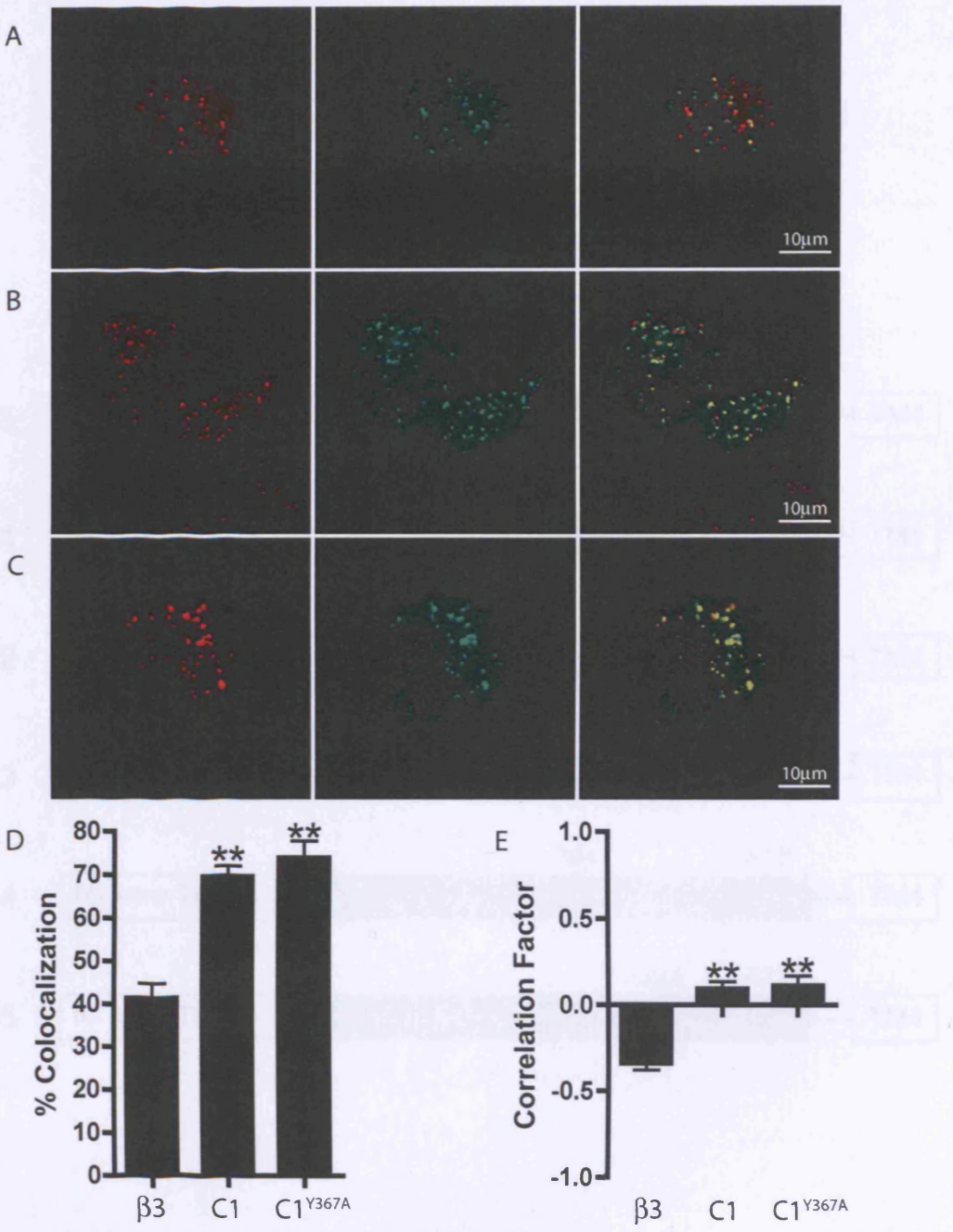


Figure 3.9: The endocytosis of C1Y367A in HEK293 cells expressing GFP-2FYVE. Antibody labelling experiments were carried out in HEK293 cells co-expressing β3 (A), C1 (B), or $C1^{Y267A}$ (C) and the early endosomal marker GFP-2FYVE. (D) Quantitative analysis shows a significant difference in the percentage of internalised $C1^{Y267A}$ (73.9 ± 4)colocalised with GFP-2FYVE compared to β3 (41.3 ± 4) but not C1 (73.83 ± 3.9). (D) This difference in the colocalisation levels between β3 and $C1^{Y367A}$ is further confirmed by a significant increase in correlation factor with GFP-2FYVE. (** P<0.01, T-test; n=29, 34, 22 for β3, C1, and $C1^{Y267A}$ respectively)

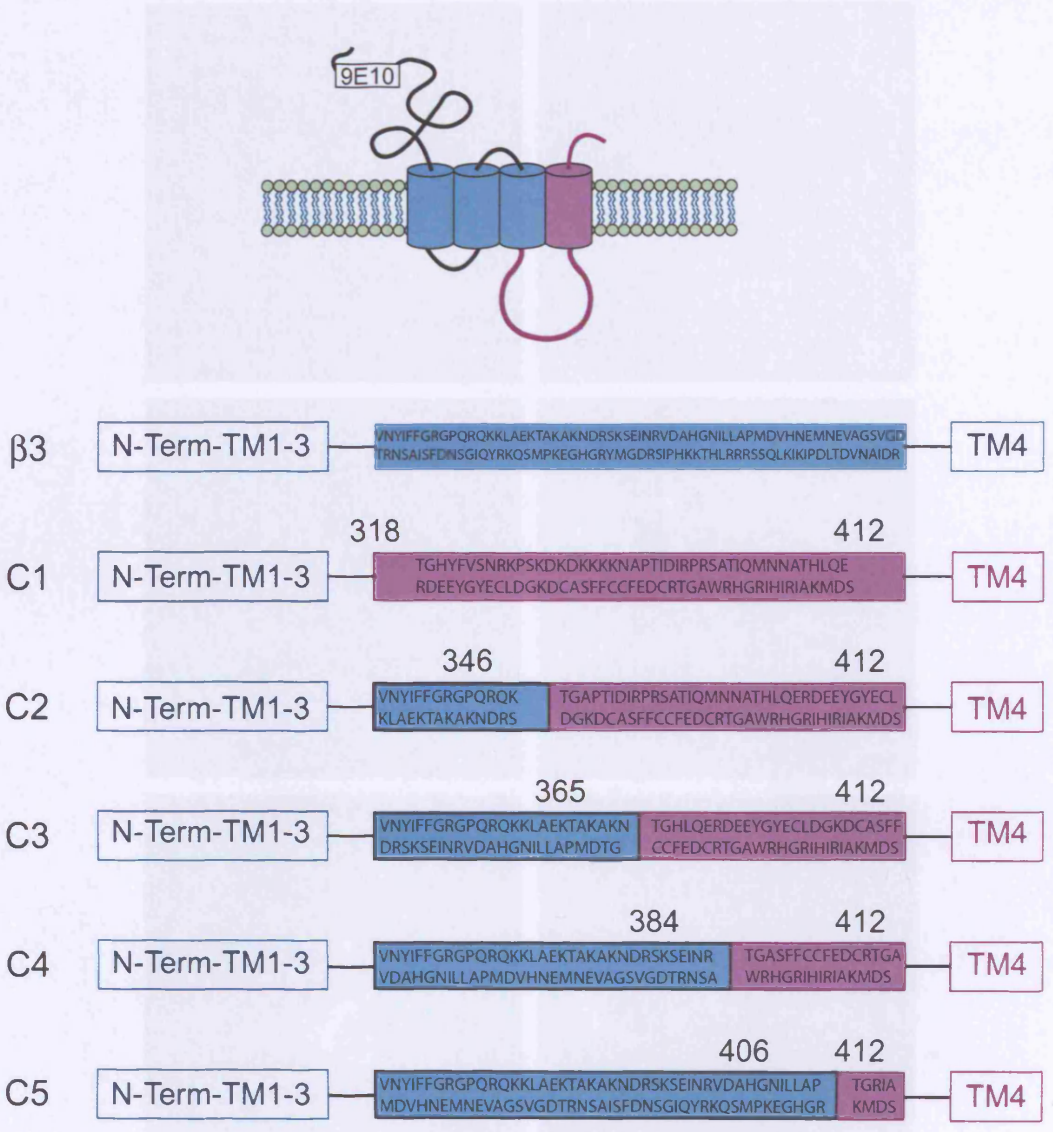


Figure 3.10: Schematic diagram of β3/γ2 chimeras. The large intracellular loop and TM4 of the $GABA_A$ receptor β3 subunit were substituted fully or partly with that of the $GABA_A$ receptor γ2 subunit.

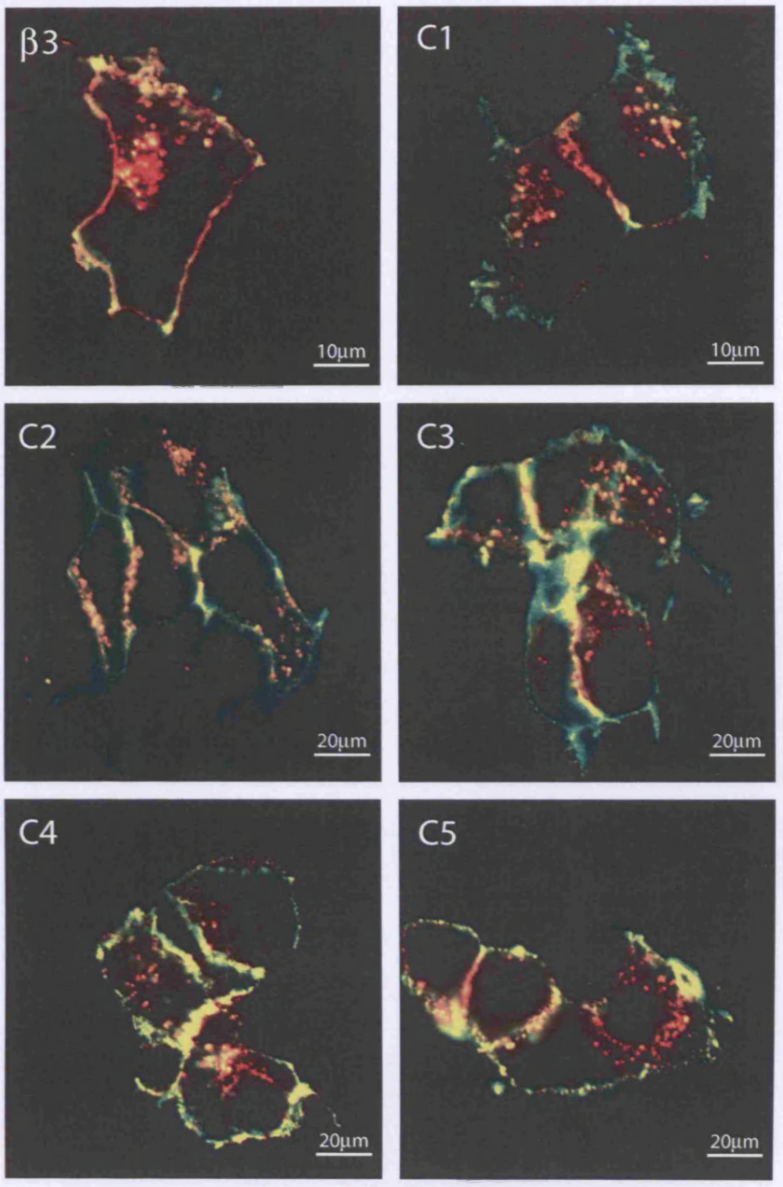


Figure 3.11: Expression of β3 and chimeras 1-5 in HEK293 cells. Antibody labelling experiments were carried out in HEK293 cells expressing ^{9E10}β3 or chimeras 1-5. Surface expression was detected in unpermeabilised cells using FITC conjugated anti mouse antibody. Internalised receptors were labelled after triton permeabilisation using Texas Red conjugated anti mouse antibody.

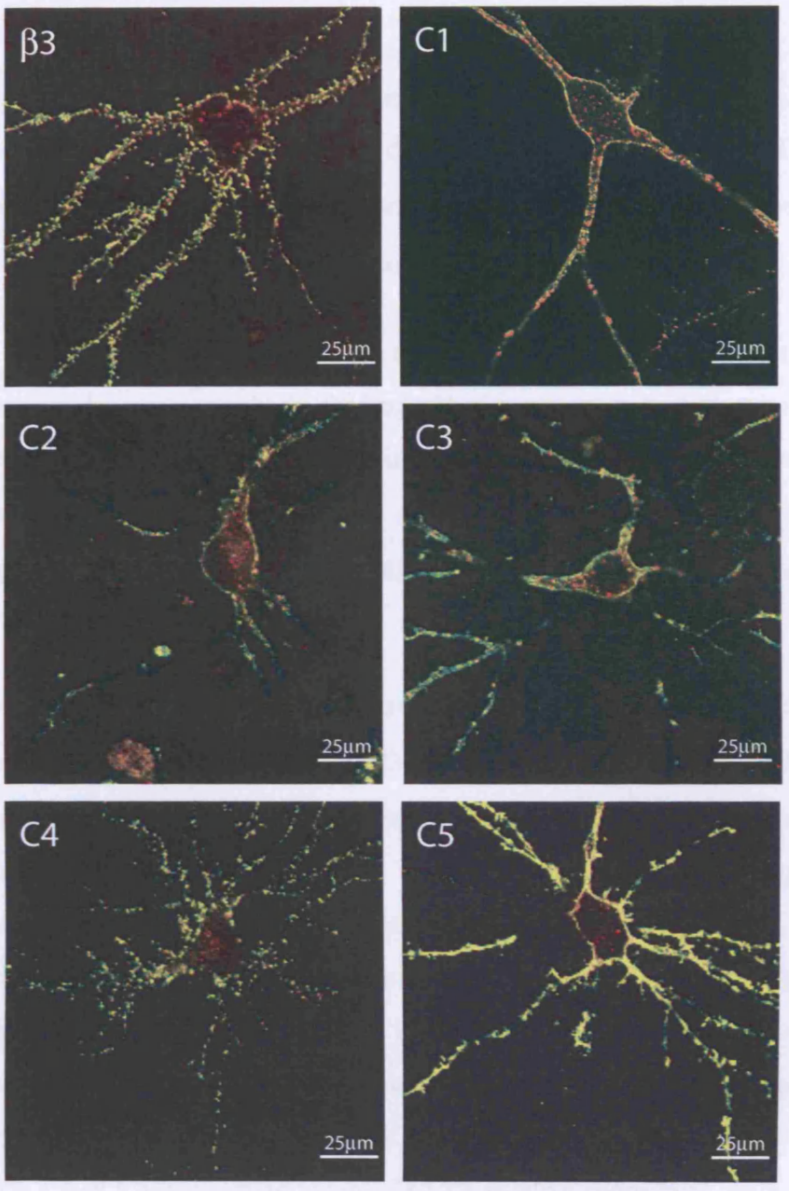


Figure 3.12: Expression of β3 and chimeras 1-5 in hippocampal neurons. Antibody feeding experiments were carried out in mature hippocampal neurons. Cell surface receptors were labelled with 9E10 anti myc antibody and allowed to internalise for 60 min. Remaining surface receptors were labelled with FITC conjugated anti mouse antibody. Internalised receptors were labelled with Texas Red conjugated antibody.

identify the possible molecular determinants of endocytic sorting of $GABA_A$ receptors four new chimeras were created. Chimeras 2-5 (C2-5) consist of amino acid substitutions where the N-terminal amino acids of the γ2 subunit intracellular loop are replaced with those of the β3 subunit (Fig. 3.10).

To test the ability of all the chimeras to access the cell surface and undergo constitutive endocytosis, antibody feeding experiments were carried out in both transfected HEK293 and cultured hippocampal neurons as shown above. Similar to the β3 subunit and C1, C2-5 were able to reach the cell surface and internalise in a constitutive manner in both HEK293 cells (Fig. 3.11) and cultured hippocampal neurons (Fig. 3.12).

In order to assess the endocytic pathway that C2-5 followed antibody feeding experiments were carried out in HEK293 co-expressing GFP-2FYVE and β3 or C1-5. After allowing surface labeled chimeras to undergo constitutive endocytosis for 30min, internalised receptors were labeled with a Texas-Red conjugated secondary antibody and imaged using confocal microscopy (Fig. 3.13). Quantitative analysis of the degree of colocalisation between the different chimeras and GFP-2FYVE revealed that unlike C1, all other chimeras created resulted in significantly ($P<0.01$) lower levels of colocalisation with GFP-2FYVE positive endosomes (Fig. 3.13 B-C). 37.7% ± 8 of C2, 38.2% ± 5 of C3, 44.3% ± 4 of C4 and 26.3% ± 4 of C5 were found in GFP-2FYVE positive endosomes. These levels of colocalisation were furthermore confirmed by significantly lower correlation factors compared to C1. However, the levels of colocalisation obtained with C2-5 were not significant compared to those obtained with internalised β3 subunits.

A 20 amino acid stretch is required for the lysosomal targeting of $GABA_A$ receptors

C1 consists of a β3 backbone and the intracellular loop of the γ2 subunit as well as TM4. In contrast C2 differs from C1 in that the first 20 amino acids of the intracellular loop of the γ2 subunit have been substituted with those of β3 (Fig.

3.10). This 20 amino acid substitution was enough to reduce the accumulation of C2 in GFP-2FYVE endosomes. Given the functional consequences of GFP-2FYVE expression and the results obtained with β3 and C1 or α1β3 and α1β3γ2L receptors, this observation indicated that these 20 amino acids may be responsible for the lysosomal targeting of γ2 subunit containing $GABA_A$ receptors. In order to confirm that C2 was not targeted for lysosomal degradation, antibody feeding experiments were carried out in HEK293 cells expressing β3, C1, or C2 and the late endosomal/lysosomal marker GFP-Rab7 (Fig. 3.14). Quantitative analysis showed that 30.2% ± 4 of internalised C2 was found in GFP-Rab7 positive endosomes. This was confirmed with a low correlation factor of -0.50 ± 0.04. These results showed a highly significant ($P<0.01$) reduction in the overlap with late endosomes of internalised C2 compared to those obtained with C1 but not β3 (Fig. 3.14).

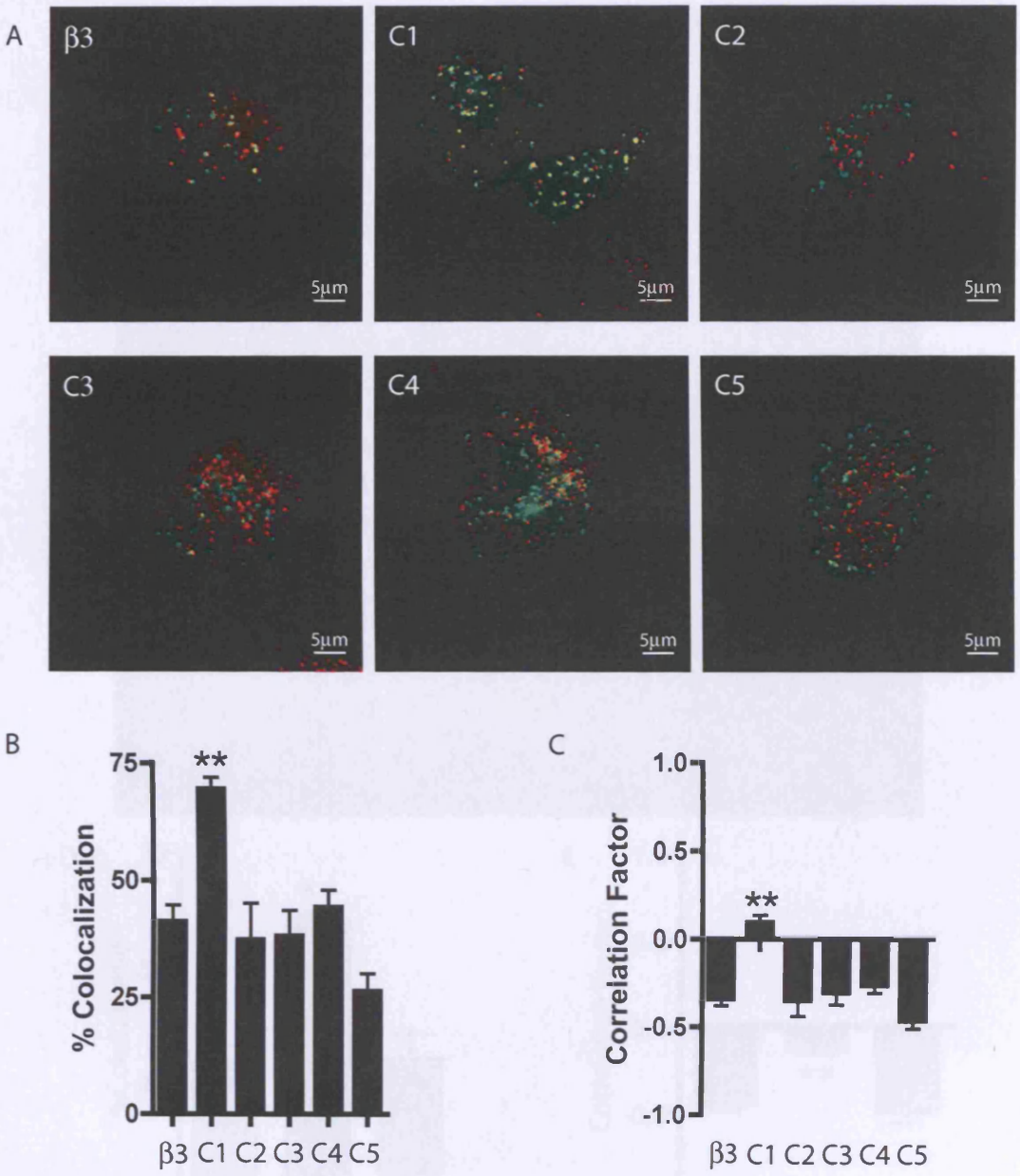


Figure 3.13: The endocytosis of β3 and C1-5 in HEK293 cells expressing GFP-2FYVE. Antibody labelling experiments were carried out in HEK293 cells co-expressing β3 or C1-5 (red) and the early endosomal marker GFP-2FYVE (A). Quantitative analysis shows a low percentage of internalised β3 and C2-5 colocalised with GFP-2FYVE positive endosomes (C) and a low correlation factor (D) compared to C1. (** P<0.01, T-test; n=29, 34, 10, 16, 10, 13 β3, C1, C2, C3, C4, C5 respectively)

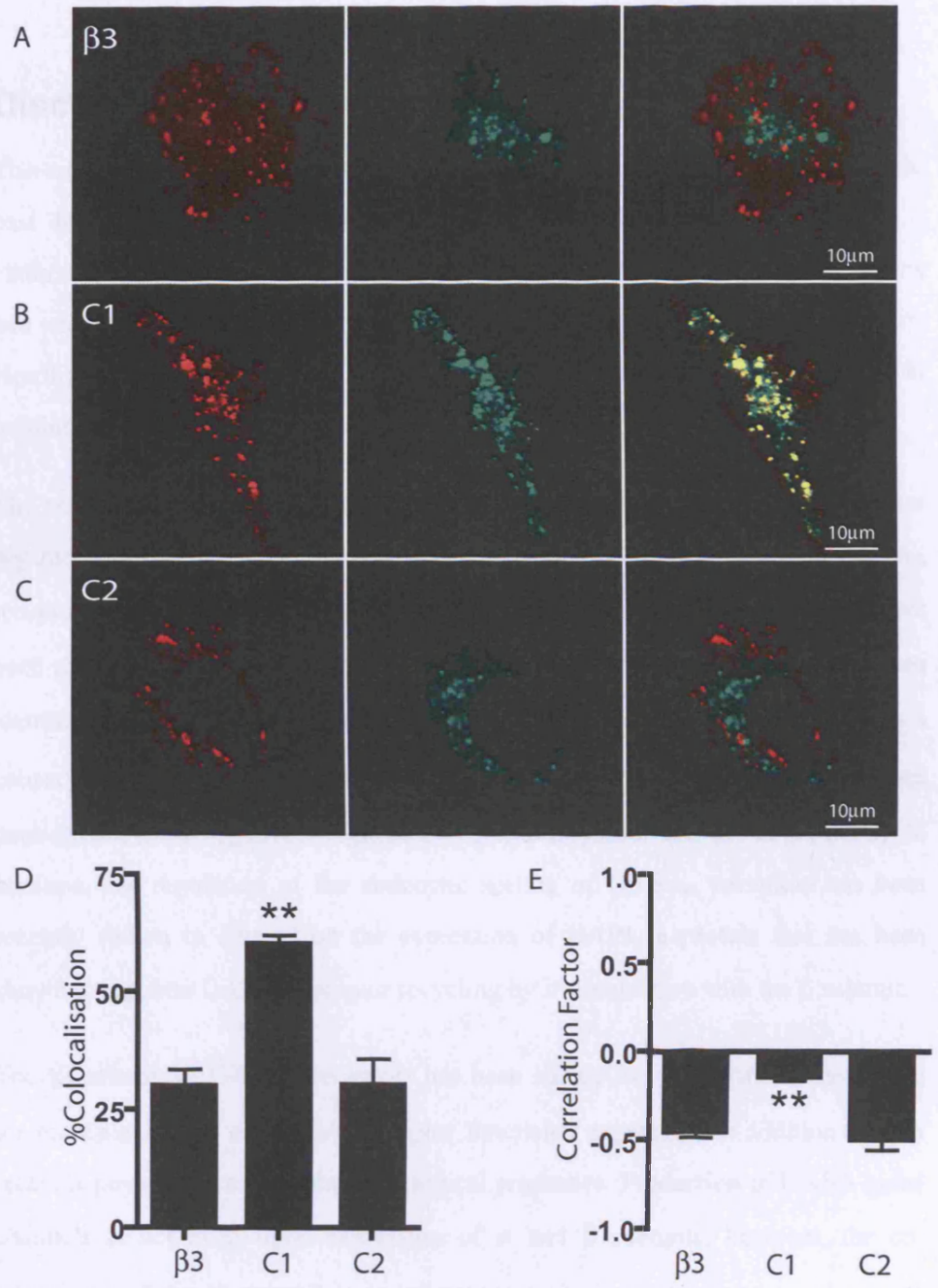


Figure 3.14: The endocytosis of β3, C1 and C2 in HEK293 cells expressing GFP-Rab7. Antibody feeding experiments were carried out in HEK293 cells co-expressing β3 (A), C1 (B) or C2 (C) and the late endosomal marker GFP-Rab7. Quantitative analysis shows a low percentage of internalised β3 and C2 colocalised with GFP-Rab7 positive endosomes (D) and a low correlation factor (E). In contrast, a high percentage of internalised C1 is found in GFP-Rab7 positive endosomes (D) and this is reflected by a significant increase in the correlation factor compared to β3 (E). (** $P<0.01$, T-test; n=12, 27, 15 for β3, C1, C2 respectively)

Discussion

The endocytosis of $GABA_A$ receptors has been an active area of research for the past decade. Multiple studies have shown $GABA_A$ receptors to internalise in a clathrin and dynamin dependent manner in both heterologous expression systems and cultured neurons (Kittler et al., 1999, 2000; van Rijnsoever et al., 2005; Herring et al., 2003). Importantly, this mechanism has been shown to play a role in regulating inhibitory synaptic strength.

The molecular mechanisms underlying the endocytosis of $GABA_A$ receptors are begininng to be understood. Interestingly the majority of data obtained on $GABA_A$ receptor endocytosis involves the β subunits. Although both β and γ subunits have been shown to bind the clathrin adaptor protein AP2, a binding site has only been identified on the β subunit (Kittler et al., 2005). Interestingly this site overlaps a conserved phosphoryolation site in all β subunits and the interaction with AP2 has been shown to be negatively regulated by phosphorylation (Kittler et al., 2005). In addition, the regulation of the endocytic sorting of $GABA_A$ receptors has been recently shown to depend on the expression of HAP1, a protein that has been shown to regulate $GABA_A$ receptor recycling by its interaction with the β subunit.

The γ2 subunit of $GABA_A$ receptors has been shown to be of critical importance for multiple aspects of $GABA_A$ receptor functional expression in addition to both receptor physiological and pharmacological properties. Production of GABA gated channels is achieved upon expression of α and β subunits, however, the co-expression of the γ2 subunit is necessary to confer benzodiazepine sensitivity to these channels and increases their single channel conductance (Pritchett et al., 1989). Furthermore it has also been proposed that the γ2 subunit is necessary for the post-synaptic targeting and clustering of $GABA_A$ receptors (Essrich et al., 1998; Alldred et al., 2004).

Despite the extensive studies on $GABA_A$ receptor endocytosis very little is known on the role that the γ2 subunit plays as a determinant of this process. Initial studies

have already suggested that the γ2 subunit may play a role in the endocytic pathway (Connolly et al., 1999; Kittler et al., 2000). However, whether this subunit directly regulates the rate of receptor endocytosis or the trafficking of $GABA_A$ receptors within the endocytic pathway remains to be established.

γ2 containing $GABA_A$ receptors have been previously reported to recycle back to the plasma membrane upon internalisation (Connolly et al., 1999). Furthermore, internalised γ2 containing $GABA_A$ receptors have been observed associated with the postsynaptic density where they overlap with gephyrin (van Rijnsoever et al., 2004). In the present study, however, a significant proportion of γ2 containing $GABA_A$ receptors were shown to be destined for lysosomal degradation. In contrast, the majority of internalised $GABA_A$ receptors lacking the γ2 subunit were not found in late endosomes and are most probably recycled back to the plasma membrane. These result suggest that the γ2 subunit contains an amino acid motif capable of conferring late endosomal/lysosomal targeting on to $GABA_A$ receptors.

In order to further investigate the molecular signals that may determine this lysosomal targeting of γ2 containing $GABA_A$ receptors a chimeric system was created. The control subunit (β3) was shown to follow a similar endocytic sorting fate as those $GABA_A$ receptors consisting of α1 and β3 subunits alone. In contrast, a chimera where the β3 subunit intracellular domain and TM4 has been exchanged for that of the γ2 subunit (C1) followed a late endosomal/lysosomal targeting pathway much like α1β3γ2 containing receptors. These results were observed in both HEK293 cells and cultured hippocampal neurons, establishing β3 and C1 as adequate representatives of α1β3 and α1β3γ2 containing receptors respectively.

The intracellular loop of the γ2 subunit contains a YXXØ sequence motif. YXXØ is a tyrosine based signal that has been implicated in the internalisation of various membrane proteins, including the NR2B subunit of NMDA receptors, by its ability to bind AP2 (Roche et al., 2001; Bonifacino and Traub, 2003). In addition, YXXØ

signals have also been implicated in further trafficking events such as lysosomal targeting from endosomes (Bonifacino and Traub, 2003; Williams and Fukuda, 1990). The possible role of this tyrosine based sorting signal found within the γ2 subunit intracellular loop was investigated using a mutated version of C1. Surprisingly, $C1^{Y367A}$ mutant was not only found to internalise, but internalised $C1^{Y367A}$ was found to accumulate in GFP-2FYVE positive endosomes. The degree of colocalisation of $C1^{Y367A}$ with GFP-2FYVE was significantly different to the levels obtained with internalised β3, but not to those obtained with internalised C1. Given the ability of GFP-2FYVE to accumulate in early endosomes internalised receptor cargo destined for late endosomes (Petiot et al., 2003), these results suggest that Y^{367} does not play a role in the late endosomal/lysosomal targeting of $GABA_A$ receptors under basal conditions in HEK293 cells.

A new set of chimeras was created in order to identify the region within the γ2 intracellular loop responsible for the late endosomal/lysosomal targeting of γ2 containing $GABA_A$ receptors. Antibody feeding experiments in HEK cells overexpressing the chimeras and GFP-2FYVE and quantitative confocal microscopy revealed that only C1 was able to behave like α1β3γ2L containing receptors and show a significant proportion of internalised receptors accumulated in GFP-2FYVE positive endosomes. These results were further confirmed using the late endosomal/lysosomal marker GFP-Rab7.

C2 showed a significant reduction in the levels of internalised receptor found in late endosomes compared to the results obtained with C1. Interestingly C1 and C2 differ in their sequence by 20 amino acids in the N-terminus of the intracellular loop. These results suggested that the first 20 amino acids of the γ2 subunit intracellular loop contained a motif required for late endosomal/lysosomal targeting.

The degree of late endosomal/lysosomal targeting achieved with γ2 containing $GABA_A$ receptors in HEK293 cells is unlikely to occur in neurons. Previous

studies have revealed that the majority of internalised γ2 containing $GABA_A$ receptors are found in the postsynaptic density where the presence of late endosomes is unlikely (van Rijnsoever et al., 2004). In addition, quantitative biotinylation experiments in neurons have reported that only a small proportion of all β3 containing $GABA_A$ receptors are degraded over time (Kittler et al., 2004b). The previously reported studies on $GABA_A$ receptor internalisation and the results obtained in this study therefore suggest the possibility that strict regulatory mechanisms may exist in neurons to specifically determine the γ2 subunit dependent endosomal sorting of internalised $GABA_A$ receptors. It would therefore be interesting to elucidate any regulatory signals within the 20 amino acid stretch responsible for the degradation of γ2 containing $GABA_A$ receptors.

4. S327 dependent ubiquitination regulates $GABA_A$ receptor late endosomal/lysosomal targeting

Introduction

In the previous section the sorting fate of internalised α1β3 and α1β3γ2L containing $GABA_A$ receptors was investigated in HEK293 cells. $GABA_A$ receptors composed of α1, β3, and γ2L subunits were found to constitutively internalise with significant proportion being targeted to late endosomes. In constrast, receptors composed of α1 and β3 subunits were also found to internalise in a constitutive manner, but the vast majority of internalised receptors were not targeted to late endosomes. These results suggest that the γ2 subunit is likely to play a specific role in regulating the late endosomal/lysosomal targeting of internalised $GABA_A$ receptors. Further analysis using β3/γ2 chimeras and quantitative confocal microscopy, allowed for the identification of a 20 amino acid region within the intracellular loop of the $GABA_A$ receptor γ2 subunit adjacent to TM3, responsible for the late endocytic targeting of $GABA_A$ receptors. Previous findings on $GABA_A$ receptor internalisation kinetics suggests that the endocytic sorting fate of $GABA_A$ receptors may be tightly regulated (Kittler et al., 2004b). However, no molecular determinants for the regulation of $GABA_A$ receptor sorting have been identified.

The endocytic sorting fate of internalised transmembrane receptors can be, in part, determined by specific amino acid motifs within the intracellular tails of these proteins. Tyrosine based signals have been identified as key internalisation motifs by their ability to bind the clathrin adaptor protein AP2 as well as important for the targeting to late endosomes from the early/sorting endosome (Bonifacino and Traub, 2003). However, the results presented in chapter 3 suggest that at least under basal conditions in HEK293 cells a tyrosine motif present in the intracellular loop of the γ2 subunit does not appear to participate in the targeting of $GABA_A$ receptors to late endosomal/lysosomal structures.

Protein ubiquitination, a process involved in the degradation of proteins in the proteasome, has been recently implicated in the sorting of cargo within the endocytic pathway in both yeast and mammalian cells. Ubiquitin has been suggested as a signal for endocytosis for a number of mammalian receptors

including the EGF receptor (Joazeiro et al., 1999; Levkowitz et al., 1999; Waterman et al., 2002), the epithelial sodium channel (Rotin et al., 2000), E-cadherin (Fujita et al., 2002) and potentially glycine receptors (Buttner et al., 2001). However, the exact mechanisms by which ubiquitination mediates receptor internalisation are not understood (DiAntonio and Hicke, 2004). Interestingly, ubiquitin has also been suggested as a signal for endocytic sorting into the lysosomal pathway. The EGF receptor (Levkowitz et al., 1998), interleukin-2 receptor (Rocca et al., 2001), CXCR4 receptor (Marchese and Benovic, 2001), β2 adrenergic receptor (Shenoy et al., 2001), growth hormone receptor (VanKerkhof and Strous, 2001) and the LDL receptor related protein (Melman et al., 2002) are all sorted into MVBs and the lysosomal pathway in a manner that is dependent of ubiquitination. Interestingly, whereas polyubiquitination appears to play a role protein degradation at the level of the proteasome, monoubiquitination is required for endocytosis and endocytic sorting (Hicke, 2001).

The $GABA_A$ receptor β3 subunit has been recently shown to be a target for ubiquitination (Saliba et al., 2005). Ubiquitination of the β3 subunit is important for regulating proteasomal degradation of GABA receptors and hence membrane insertion. Conversely, ubiquitination of this subunit was not found to be involved in the endocytic pathway. However, the possible ubiquitination of other subunits, including the γ2 subunit, has not yet been investigated.

$GABA_A$ receptor phosphorylation has been implicated in various mechanisms of receptor modulation including altering channel kinetics, open time, rate of desensitization, sensitivity to pharmacological agents as well as channel receptor stability (Brandon et al., 2002; Kittler and Moss, 2003, Kittler et al., 2004b). The large intracellular loop of $GABA_A$ receptor subunits has been shown to be the target for various protein kinases including PKC, PKA, PKG, CamKII, and Src kinase both *in vitro* and *in vivo* (Brandon et al., 2002; Kittler and Moss, 2003). In particular, the γ2 subunit has been shown to be phosphorylated *in vitro* on residues S327 and S343 by PKC (Kellenberger et al., 1992; Krishek et al., 1994) and Y365

and Y367 by Src kinase (Moss et al., 1995). Importantly, the phosphorylation of these residues in heterologous expression systems have produced varied and contradicting results ranging from an enhancement in the response to GABA to a downregulation of $GABA_A$ receptor function. While these results may reflect a specific role for individual kinase isoforms in regulating $GABA_A$ receptor membrane trafficking (Song and Messing, 2005), it should be noted that the majority of these functional studies are performed at room temperature (18-24°C), conditions in which endocytosis and intracellular membrane transport events are severely compromised.

Having established a role for the γ2 subunit in the endocytic sorting of $GABA_A$ receptors and identified a 20 amino acid motif responsible for this targeting, this chapter investigates the possible molecular determinants within these 20 amino acids in the endocytosis of $GABA_A$ receptors.

Results

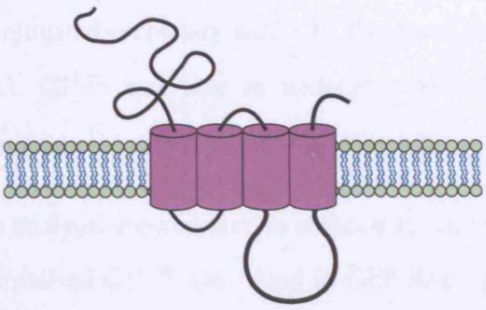
The γ2 subunit intracellular loop

The large intracellular loop between TM3 and TM4 of $GABA_A$ receptor subunits is the site for various protein interactions as well as the site for a number of post-translational modifications including phosphorylation, palmitoylation and ubiquitination (Brandon et al., 2002; Kittler et al., 2003; Rathenberg et al., 2003; Saliba et al., 2005). In the previous chapter, a 20 amino acid region within the γ2 subunit intracellular loop was identified to contain the molecular information for the enhanced late endosomal/lysosomal targeting γ2 subunit containing $GABA_A$ receptors in HEK293 cells under basal conditions. The aim of the following work was to attempt a better mechanistic understanding of the role of this region in the late endosomal/lysosomal targeting of $GABA_A$ receptors.

Analysis of the residues present in this 20 amino acid region within the γ2 subunit, highlights two interesting observations: Firstly, this region is heavily rich in lysine residues (Fig. 4.1 B (red)); secondly, this region also contains residue S327 a known target for phosphorylation (Fig. 4.1 B (blue)). This suggested that ubiquitination and phosphorylation within this region may be important for $GABA_A$ receptor sorting. In order to investigate the possible role of these residues in the late endosomal/lysosomal targeting of $GABA_A$ receptors, antibody labeling experiments were carried out in HEK293 cells expressing mutant versions C1 and heteromeric receptors.

A stretch of lysines regulates $GABA_A$ receptor sorting

In order to investigate the possible role of a stretch of lysine residues in the intracellular loop of the γ2 subunit in receptor endocytic sorting, site directed mutagenesis was carried out in C1. All 7 lysines (K325, 328, 330, 332, 333, 334 and 335) present in the first 20 amino acid region of the intracellular loop were replaced with arginine, which is a conservative substitution; and the resulting construct was termed $C1^{K7R}$.



HYFVSNRKPSKDKDKKKKNPLLRMFSFKAPTIDIRPRSATIQMNNATH
LQERDEEYGYECLDGKDCASFFCCFEDCRTGAWRHGRIHIRIAKMDS

Figure 4.1: The intracellular loop of the γ2 subunit. (A) Schematic diagram of the γ2 subunit. (B) Amino acid sequence of the intracellular loop of the γ2 subunit. 20 N-terminal amino acids of the intracellular loop are highllighted in light blue. Putative ubiquitination targets are highlighted in red. S327, a known phosphorylation site, is highlighted in blue.

Antibody labeling experiments were carried out in HEK293 cells co-expressing the late endosomal/lysosomal marker GFP-Rab7 and β3, C1, or $C1^{K7R}$. Labeled surface receptors were allowed to internalise at 37°C for 60min. Remaining surface receptors were stripped in an acid wash, and internalised receptors were detected using a Texas-Red conjugated secondary antibody. Confocal analysis revealed that similar to C1 and β3, $C1^{K7R}$ was able to undergo constitutive endocytosis as observed by the red punctate intracellular staining (Fig. 4.2).

However, quantitative analysis showed that, in contrast to internalised C1 (60.2% ± 4), 36.0% ± 5 of internalised $C1^{K7R}$ was found in GFP-Rab7 positive endosomes. The correlation factor between the GFP expression and internalised $C1^{K7R}$ was -0.37 ± 0.05 (Fig. 4.2). These levels of colocalisation were significantly ($P<0.001$) lower than those obtained with C1 but not compared to those obtained with the β3 subunit (Fig. 4.2).

In order to investigate whether this mutation may play a role in the endocytic sorting of fully assembled heteromeric $GABA_A$ receptors, the same 7 lysines in the intracellular loop of the γ2L subunit were mutated to arginines by site directed mutagenesis. Antibody labeling experiments were carried out in HEK293 cells co-expressing GFP-Rab7 and $^{9E10}\alpha1\beta3$; $\alpha1\beta3^{9E10}\gamma2L$ or; $\alpha1\beta3^{9E10}\gamma2L^{K7R}$ subunit combinations.

Quantitative colocalisation measurements on confocal microscopy images revealed that internalised α1β3γ2L containing receptors showed high levels of colocalisation (67.7% ± 3; correlation factor -0.1 ± 0.04) with the late endosomal/lysosomal marker GFP-Rab7 (Fig. 4.3). In contrast, there was a significant ($P<0.01$) decrease in the percentage of internalised $\alpha1\beta3\gamma2L^{K7R}$ containing receptors found in GFP-Rab7 positive endosomes (32.9 ±7) as well as a significant decrease in the correlation factor (-0.4 ± 0.1) compared to results obtained with α1β3γ2L containing receptors (Fig. 4.3). Similar to the results obtained with the chimeric system, the degree of colocalisation between internalised $\alpha1\beta3\gamma2L^{K7R}$ and GFP-

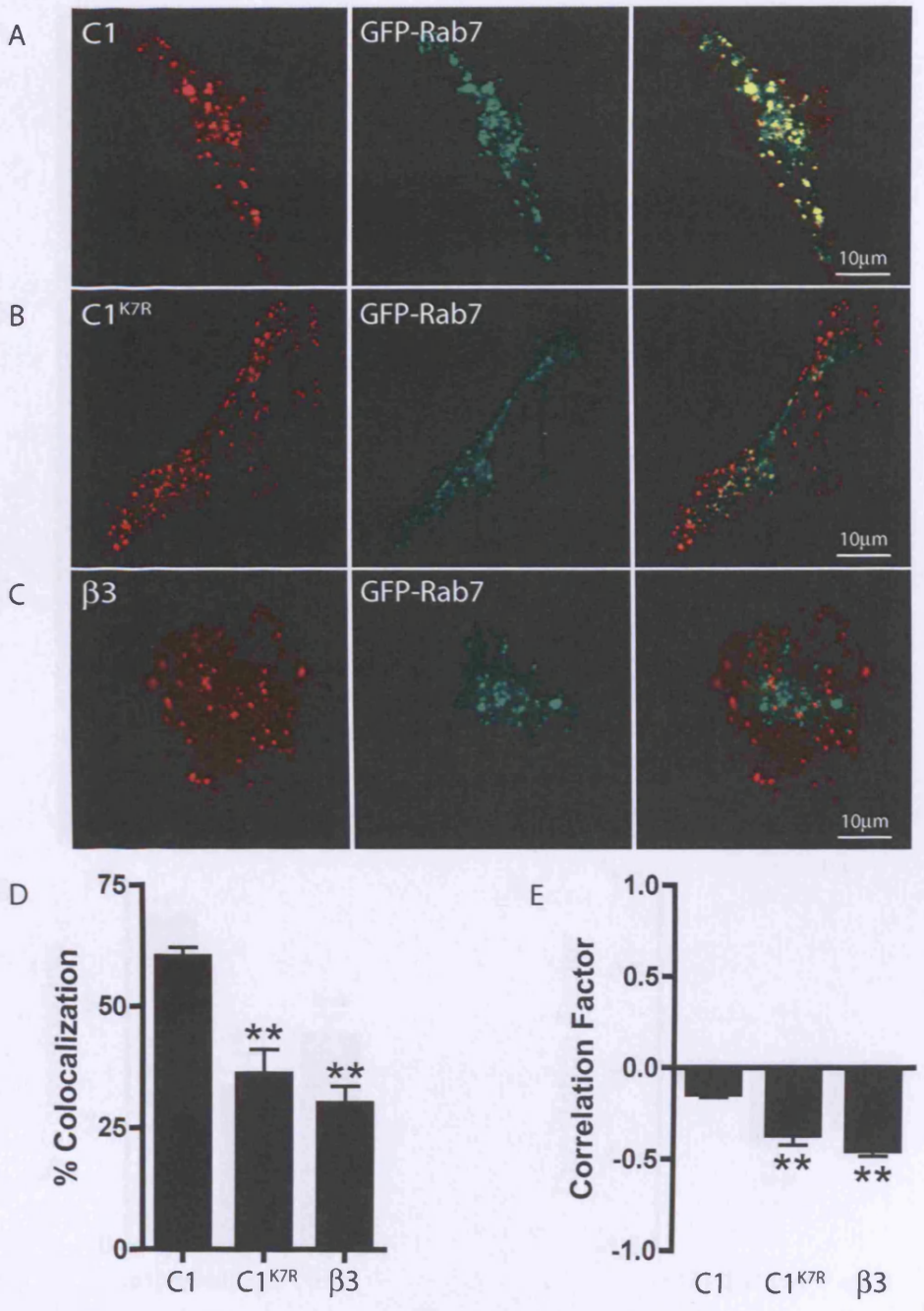


Figure 4.2: The endocytosis of C1 and $C1^{K7R}$ in HEK293 cells expressing GFP-Rab7. Antibody labelling experiments were carried out in HEK293 cells co-expressing C1 (A), $C1^{K7R}$ (B) or β3 (C) and the late endosomal marker GFP-Rab 7. (D, E) Quantitative analysis shows 60.15 % ± 2 of internalised C1 colocalised with GFP-Rab7 positive endosomes and the two channels exhibit a correlation factor of -0.14 ± 0.03. In contrast, 36.0% ± 5 of internalised $C1^{K7R}$ is found in GFP-Rab 7 positive endosomes and this is reflected by a significant decrease (- 0.22) in the correlation factor compared to C1. However, the results obtained with $C1^{K7R}$ were not siginificantly different to those obtained with the β3 subunit. (** P<0.01, T-test; n=27, 15, 12 for C1, $C1^{K7R}$ and β3 respectively.)

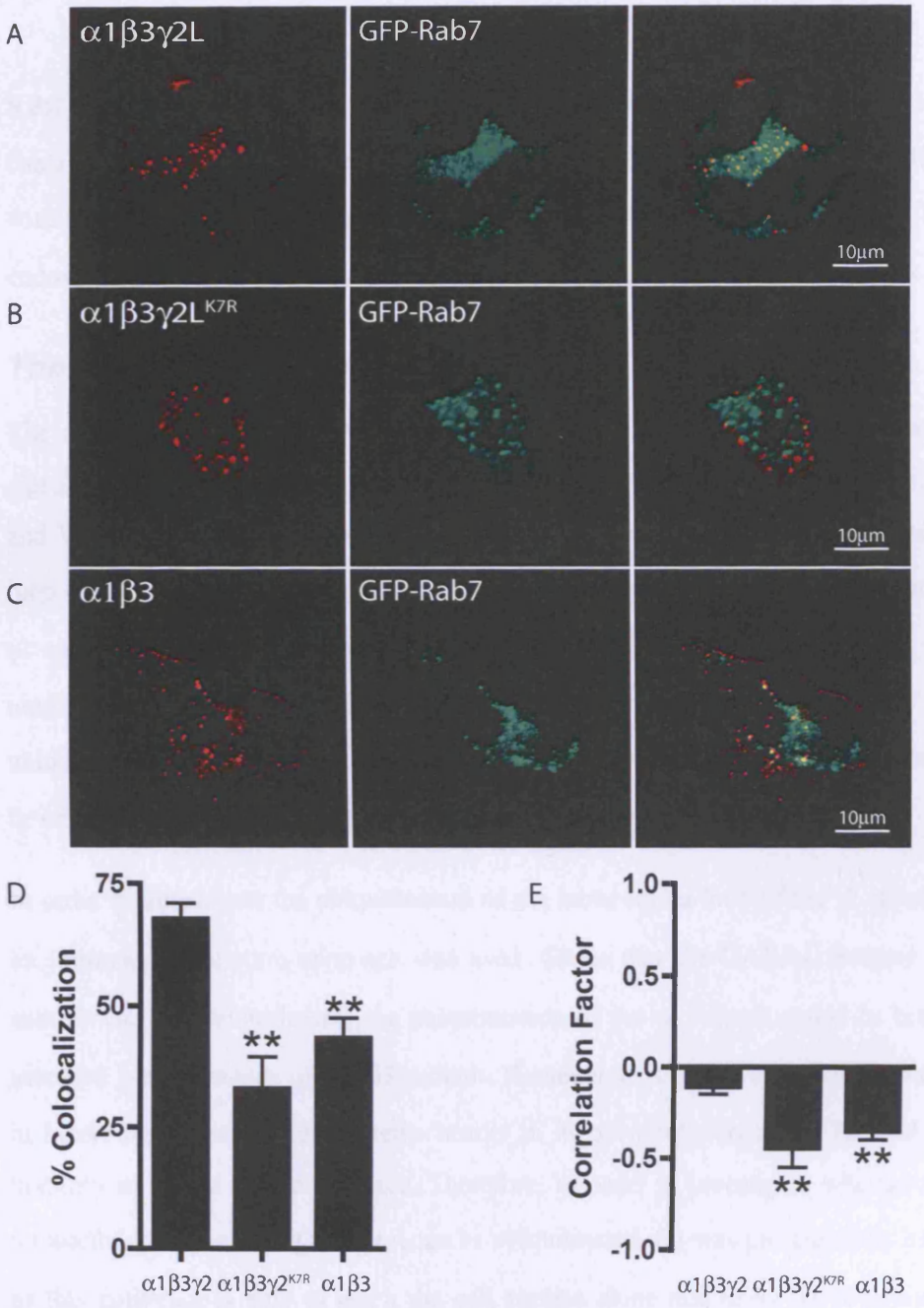


Figure 4.3: The endocytosis of $\alpha1\beta3\gamma2L^{K7R}$ in HEK293 cells expressing GFP-Rab7. Antibody labelling experiments were carried out in HEK293 cells co-expressing $\alpha1\beta3\gamma2L$ (A), $\alpha1\beta3\gamma2L^{K7R}$ (B) or $\alpha1\beta3$ (C) and the late endosomal marker GFP-Rab 7. (D, E) Quantitative analysis shows 67.7 % ± 3 of internalised $\alpha1\beta3\gamma2L$ colocalised with GFP-Rab7 positive endosomes and the two channels exhibit a correlation factor of -0.11 ± 0.04. In contrast, 32.9% ± 7 of internalised $\alpha1\beta3\gamma2L^{K7R}$ is found in GFP-Rab 7 positive endosomes and this is reflected by a significant decrease (- 0.34) in the correlation factor compared to $\alpha1\beta3\gamma2L$. However, the results obtained with $\alpha1\beta3\gamma2L^{K7R}$ were not siginificantly different to those obtained with the $\alpha1\beta3$ subunit. (** P<0.01, T-test; n=26, 7, 16 fo $\alpha1\beta3\gamma2L$, $\alpha1\beta3\gamma2L^{K7R}$ and $\alpha1\beta3$ respectively.)

Rab7 were not significantly different to those obtained with internalised α1β3 containing receptors. Together these results suggest that these 7 lysine residues within the intracellular loop of the γ2L subunit are responsible for the late endosomal/lysosomal targeting of internalised αβγ containing $GABA_A$ receptors.

The γ2 subunit is ubiquitinated

The modification of lysines by the polypeptide ubiquitin has been described as a signal for degradation in the proteasome and the lysosome (Hicke, 1999; Aguilar and Wendland, 2003). The revelation that a lysine rich stretch in the intracellular loop of the γ2 subunit is responsible for the late endosomal/lysosomal targeting of γ2 containing $GABA_A$ receptors suggested that this subunit may be a target for ubiquitination. $GABA_A$ receptors have recently been shown to be a substrate for ubiquitination on their β3 subunit, although this ubiquitination has been shown to be of importance only within the secretory pathway (Saliba et al., 2005).

In order to investigate the ubiquitination of the intracellular loop of the γ2 subunit, an immunoprecipitation approach was used. Given that the $GABA_A$ receptor β3 subunit can be ubiquitinated, the ubiquitination of the γ2 subunit would be better assessed in the absence of the β3 subunit. However, expression of the γ2L subunit in heterologous expression systems results in its accumulation in the ER and its inability to access the cell surface. Therefore, in order to investigate whether the intracellular loop of the γ2 subunit can be ubiquitinated, C1 was preferentially used as this construct is able to reach the cell surface alone due to its β3 N-terminal backbone (Fig. 3.3; Fig. 3.4).

COS cells expressing HA tagged ubiquitin (HA-Ub) and C1 or $C1^{K7R}$ were scraped in lysis buffer and sonicated. Membranes were solubilised by addition of SDS to a final concentration of 0.2% and heated to 60°C. Any remaining insoluble membranes were solubilised by the addition of triton to a final concentration of 1% and further sonication. Remaining insoluble cellular and nuclear debris was

removed by centrifugation. 10% of the supernatant was used for protein expression analysis whereas the remaining 90% was used for immunoprecipitating C1 or $C1^{K7R}$ with anti-myc antibody. Precipitates were then resolved using SDS-PAGE and analysed by western blotting. Importantly, the use of HA-Ub allows the detection of covalent modification by ubiquitin using antibodies to HA as previously described (Fujita et al., 2002).

Immunoblotting for the presence of HA-Ub in the immunoprecipitated C1 samples revealed a high molecular weight smear characteristic of ubiquitinated proteins (Fig. 4.4) suggesting that C1 is in fact a substrate for ubiquitination. Interestingly, the precipitation of $C1^{K7R}$ revelaed a highly significant ($P<0.01$) decrease in the levels of ubiquitin as determined by densitometry. These results suggest that these 7 lysines in the intracellular loop of the γ2 subunit are found ubiquitinated in HEK293 cells.

S327 can also regulate the endocytic sorting of $GABA_A$ receptors

The results presented above demonstrate a role for the γ2 subunit in the late endosomal/lysosomal targeting of $GABA_A$ receptors. Furthermore a lysine stretch is shown to be ubiquitinated and be responsible for the late endosomal/lysosomal targeting of $GABA_A$ receptors. In addition to the lysine stretch described above, another putative signal for the membrane sorting of γ2 subunit containing $GABA_A$ receptors is S327.

Given the location of S327 within the endocytic sorting signal of the γ2 intracellular loop and its possible functional modulation of $GABA_A$ receptor expression, it is possible that S327 may also play a role in the endocytic sorting of γ2 containing $GABA_A$ receptors. Below, a mutant version of C1 ($C1^{S327A}$) is used in antibody labeling experiments to investigate the possible role of this residue in the late endosomal/lysosomal targeting of γ2 subunit containing $GABA_A$ receptors.

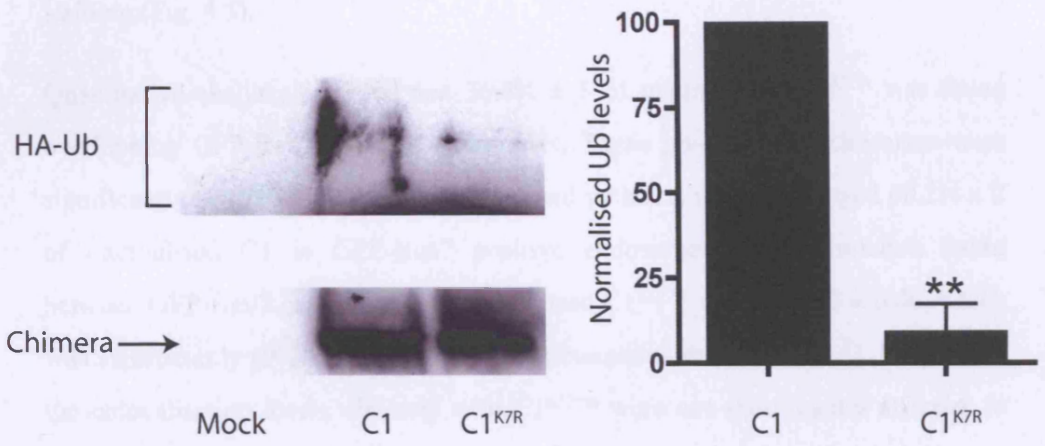


Figure 4.4: Ubiquitination of C1. Lysates of COS cells expressing HA-Ub alone, or co-expressed with C1 or $C1^{K7R}$ were immunoprecipitated with anti-9E10 antibody and immunoblotted against HA. HA levels were quantified by densitometry and normalised to total expression of the chimeras with levels in control (C1) assigned a value of 100%. (** $P<0.01$, T-test; n=3)

Antibody labeling experiments were carried out in HEK293 cells co-expressing the late endosomal/lysosomal marker GFP-Rab7 and $^{9E10}\beta3$, C1, or $C1^{S327A}$. Labeled surface receptors were allowed to internalise at 37°C for 60min. Remaining surface receptors were stripped in an acid wash, and internalised receptors were detected using a Texas-Red conjugated secondary antibody. Similar to the results obtained with $C1^{K7R}$, confocal analysis revealed that similar to C1 and β3, $C1^{S327A}$ was able to undergo constitutive endocytosis as observed by the red punctate intracellular staining (Fig. 4.5).

Quantitative analysis revealed that 36.6% ± 5 of internalised $C1^{S327A}$ was found overlapping GFP-Rab7 positive endosomes. These levels of colocalisation were significantly ($P<0.01$) lower to those obtained with C1, which displayed 60.2% ± 2 of internalised C1 in GFP-Rab7 positive endosomes. The correlation factor between GFP-Rab7 expression and internalised $C1^{S327A}$ was of -0.45 ± 0.06, which was significantly ($P<0.01$) lower than that obtained with C1 (Fig. 4.5). In contrast, the colocalisation levels obtained with $C1^{S327A}$ were not significantly different to those obtained with internalised β3 subunit (Fig. 4.5).

In order to investigate whether this mutation could also play a role in the endocytic sorting of fully assembled heteromeric $GABA_A$ receptors, S327 in the intracellular loop of the γ2L subunit was mutated to an alanine residue by site directed mutagenesis. Antibody labeling experiments were carried out in HEK293 cells co-expressing GFP-Rab7 and $^{9E10}\alpha1\beta3$; $\alpha1\beta3^{9E10}\gamma2L$ or; $\alpha1\beta3^{9E10}\gamma2L^{S327A}$ subunit combinations.

Quantitative colocalisation measurements on confocal microscopy images revealed that internalised α1β3γ2L containing receptors showed high levels of colocalisation (67.7% ± 3; correlation factor -0.11 ± 0.04) with the late endosomal/lysosomal marker GFP-Rab7 (Fig. 4.6). In contrast, there was a significant ($P<0.01$) decrease in the percentage of internalised $\alpha1\beta3\gamma2L^{S327A}$ containing receptors found in GFP-Rab7 positive endosomes (43.6 ± 4) as well as a significant decrease in the

correlation factor (-0.37 ± 0.04) compared to results obtained with α1β3γ2L containing receptors (Fig. 4.6). Similar to the results obtained with the chimeric system, the degree of colocalisation between internalised $\alpha1\beta3\gamma2L^{S327A}$ and GFP-Rab7 were not significantly different to those obtained with internalised α1β3 containing receptors. Together these results suggest that S327 is required for the late endosomal/lysosomal targeting of internalised αβγ containing $GABA_A$ receptors.

S327 is necessary for the ubiquitination of $GABA_A$ receptors

The results presented above suggest that the late endosomal/lysosomal targeting of γ2 containing $GABA_A$ receptors is dependent on a lysine stretch within the intracellular loop of the γ2 subunit. In addition this lysine stretch is a substrate for ubiquitination, a known signal for endocytic membrane sorting. Furthermore, the presence of S327, a substrate for PKC dependent phosphorylation, is also required for the late endosomal/lysosomal targeting of γ2 containing $GABA_A$ receptors. However, whether these two signals act in concert or independently of each other is unclear.

The ubiquitination of lysines occurs in three enzyme mediated steps: activation, conjugation and ligation. Substrate recognition is carried out by the ligase enzyme type E3 (see introduction, Fig. 1.3). How E3 ligases recognize their targets varies can vary among different proteins. However, there are reports for a number of E3 ligases where substrate recognition occurs by binding phosphorylated (Yaron et al., 1998; Skowyra et al., 1997; Feldman et al., 1997) or dephosphorylated (Honda and Yasuda, 1999) residues. Given the role of S327 in the late endosomal/lysosomal targeting of γ2 containing $GABA_A$ receptors and its previously characterised effects on GABAergic function, it may be possible that S327 can regulate the ubiquitination of the lysine stretch in the intracellular loop of the γ2 subunit.

In order to investigate the possible role of S327 in the ubiquitination of the intracellular loop of the γ2 subunit COS cells expressing HA tagged ubiquitin and

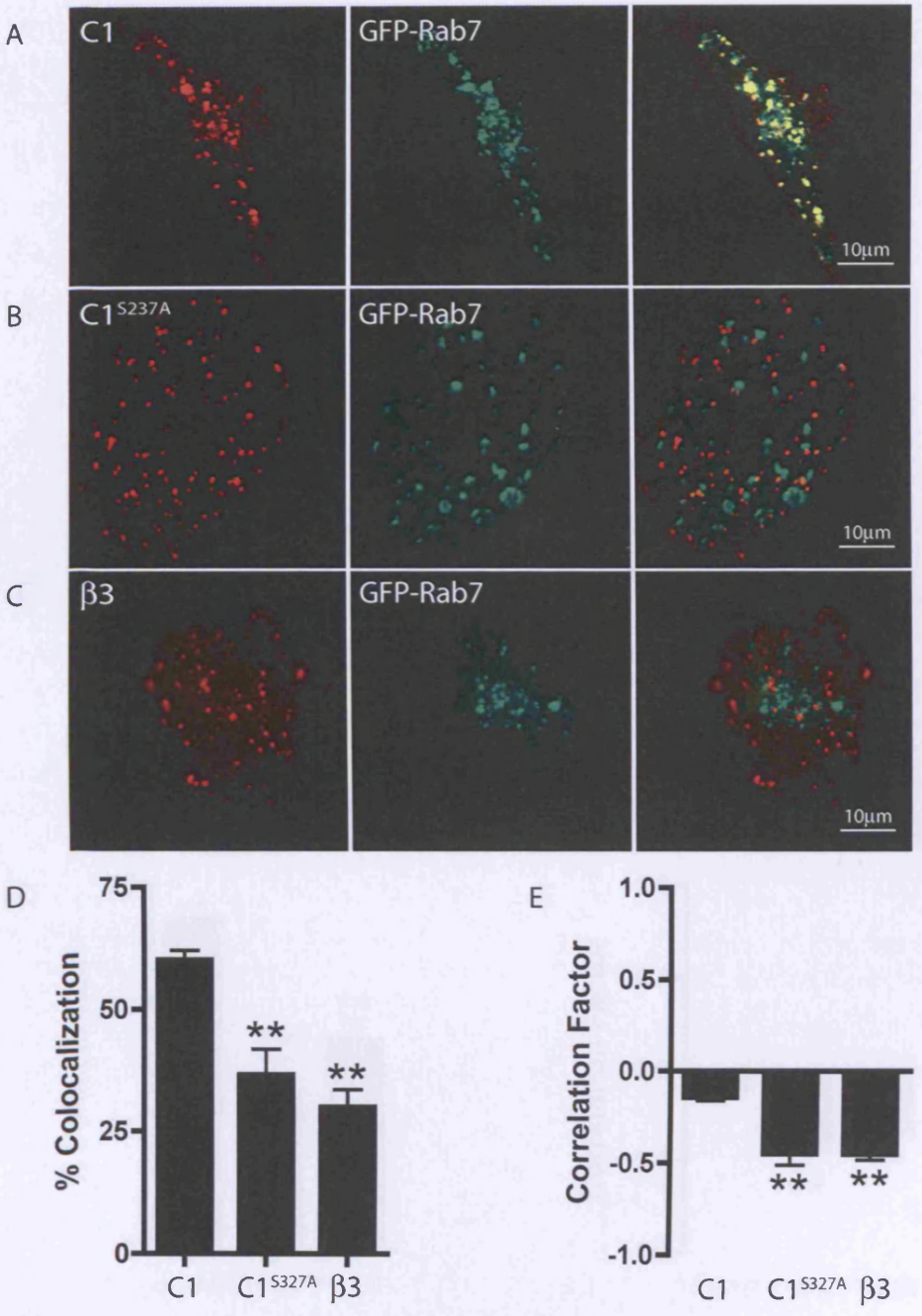


Figure 4.5: The endocytosis of C1 and $C1^{S327A}$ in HEK293 cells expressing GFP-Rab7. Antibody labelling experiments were carried out in HEK293 cells co-expressing C1 (A), $C1^{S327A}$ (B) or β3 (C) and the late endosomal marker GFP-Rab 7. (D, E) Quantitative analysis shows 60.2 % ± 4 of internalised C1 colocalised with GFP-Rab7 positive endosomes and the two channels exhibit a correlation factor of -0.14 ± 0.03. In contrast, 36.6% ± 5 of internalised $C1^{S327A}$ is found in GFP-Rab 7 positive endosomes and this is reflected by a significant decrease (- 0.31) in the correlation factor compared to C1. However, the results obtained with $C1^{S327A}$ were not siginificantly different to those obtained with the β3 subunit. (** $P<0.01$, T-test; n=27, 16, 12 for C1, $C1^{S327A}$ and β3 respectively.)

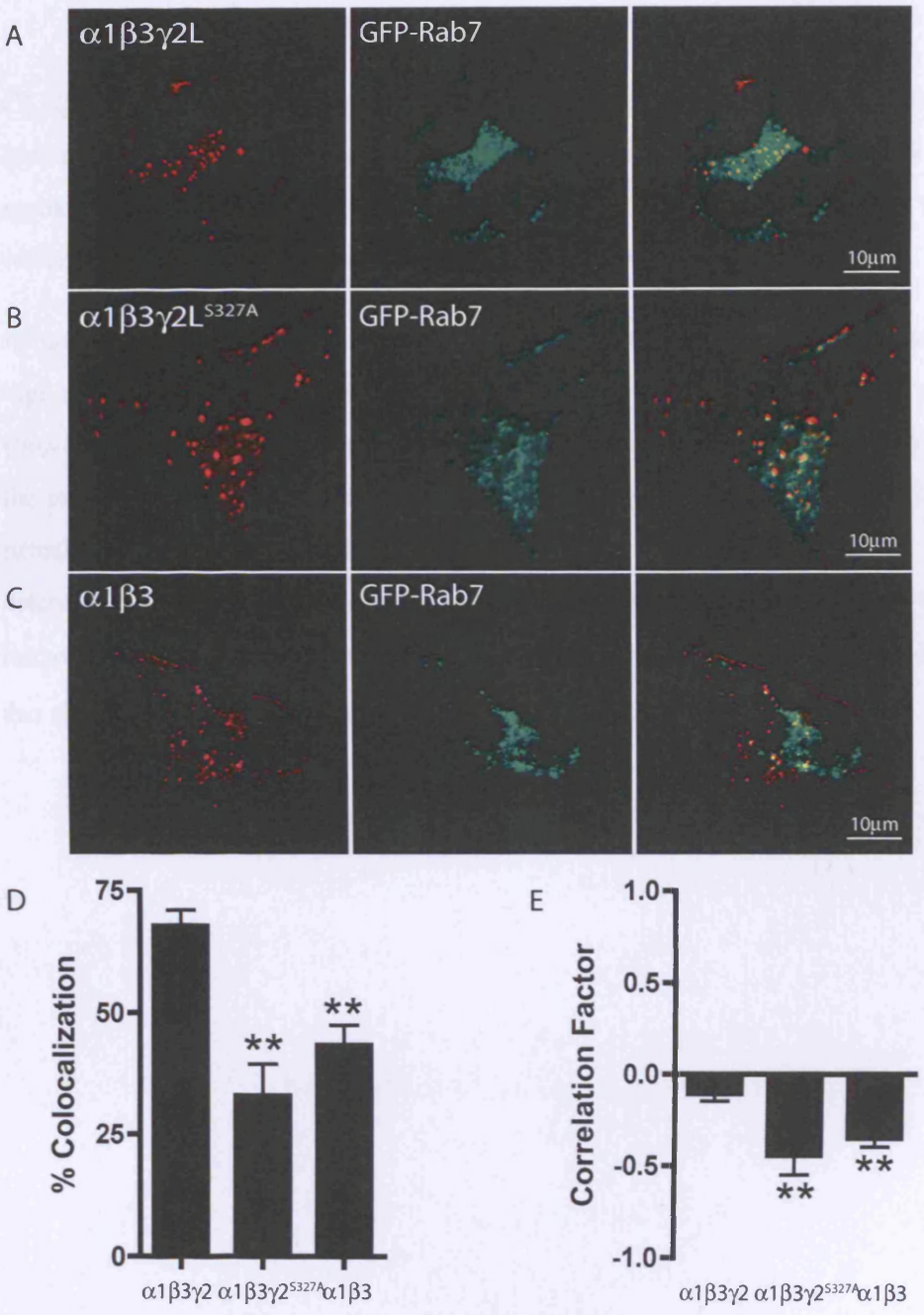


Figure 4.6: The endocytosis of α1β3γ2L^{S327A} in HEK293 cells expressing GFP-Rab7. Antibody labelling experiments were carried out in HEK293 cells co-expressing α1β3γ2L (A), α1β3γ2L^{S327A} (B) or α1β3 (C) and the late endosomal marker GFP-Rab 7. (D, E) Quantitative analysis shows 67.7 % ± 4 of internalised α1β3γ2L colocalised with GFP-Rab7 positive endosomes and the two channels exhibit a correlation factor of -0.11 ± 0.04. In contrast, 43.6% ± 4 of internalised α1β3γ2L^{S327A} is found in GFP-Rab 7 positive endosomes and this is reflected by a significant decrease (- 0.26) in the correlation factor compared to α1β3γ2L. However, the results obtained with α1β3γ2L^{S327A} were not siginificantly different to those obtained with the α1β3 subunit. (** P<0.01, T-test; n=26, 11, 16 for α1β3γ2L, α1β3γ2L^{S327A} and α1β3 respectively.)

C1, $C1^{K7R}$ or $C1^{S327A}$ were lysed by scraping and sonication in SDS and Triton buffer. Lysates were immunoprecipitated with anti-myc antibody and protein A sepharose. Precipitates were then resolved using SDS-PAGE and analysed by western blotting using monoclonal anti-HA (12CA5) antibody.

Immunoblotting for the presence of HA-Ub in C1 precipitated samples revealed a high molecular weight smear characteristic of ubiquitinated proteins (Fig. 4.7). As shown above, the ubquitination levels were significantly reduced when 7 lysines in the intracellular loop were mutated. Interestingly, the precipitation of $C1^{S327A}$ also revealed a highly significant ($P<0.01$) decrease in the levels of ubiquitin as determined by densitometry. These results suggest that these 7 lysines in the intracellular loop of the γ2 subunit are found ubiquitinated in HEK293 cells and this ubiquitination is dependent on the presence of S327.

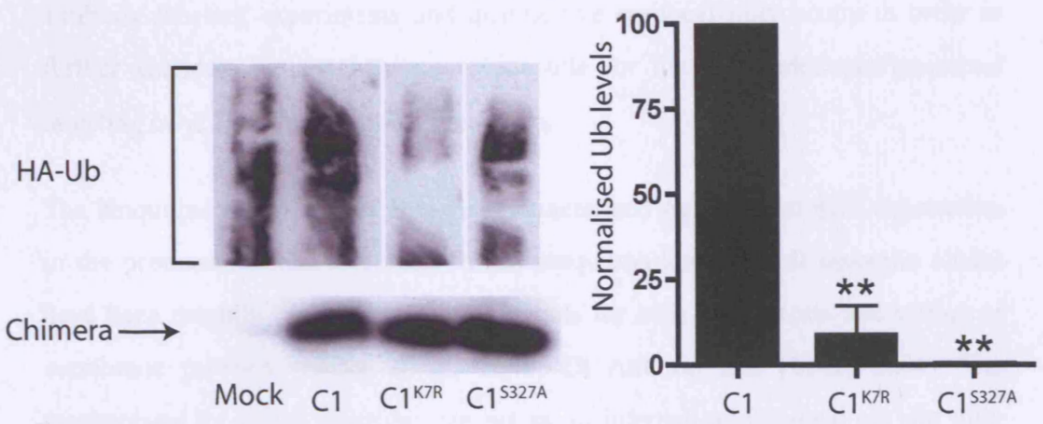


Figure 4.7: S327 dependent ubiquitination of C1. Lysates of COS cells expressing HA-Ub alone, or co-expressed with C1, $C1^{K7R}$ or $C1^{S327A}$ were immunoprecipitated with anti-9E10 antibody and immunoblotted against HA. HA levels were quantified by densitometry and normalised to total expression of the chimeras with levels in control (C1) assigned a value of 100%. (** $P<0.01$, T-test; n=3).

Discussion

The endocytic sorting of $GABA_A$ receptors is, in part, determined by subunit composition. The work presented in this thesis, demonstrates a critical role for the γ2 subunit in the late endosomal/lysosomal targeting of internalised $GABA_A$ receptors. Furthermore, using a chimeric system, a 20 amino acid region responsible for the late endosomal/lysosomal targeting of $GABA_A$ receptors was identified. In this chapter, site directed mutagenesis was used together with antibody labeling experiments and quantitative confocal microscopy in order to further elucidate the mechanisms responsible for the late endosomal/lysosomal targeting of γ2 containing $GABA_A$ receptors.

The ubiquitination of lysines is a well characterised signal for protein degradation in the proteasome. More recently, mono-ubiquitination or small ubiquitin chains have been recently identified to act as signals for both endocytosis and sorting of membrane proteins (Hicke et al., 2001; Di Antonio and Hicke, 2004). The mechanisms by which ubiquitin can act as an internalisation signal are not fully understood. In constrast, a number of proteins of the endocytic sorting machinery have been shown to contain ubiquitin interacting motifs (UIM), and it is thought that the binding of these motifs to ubiquitin facilitates the trafficking of ubiquitinated proteins to late endosomes and consequently lysosomal degradation (Dupre et al., 2001; Rocca et al., 2001).

Sequence analysis of the intracellular loop of the γ2 subunit revealed the presence of multiple lysine residues within the 20 amino acid region thought to contain a signal for late endosomal/lysosomal targeting of $GABA_A$ receptors. All 7 lysines within this region were mutated by site directed mutagenesis to arginine residues in both C1 and the γ2L $GABA_A$ receptor subunit. These mutations resulted in a reduction in the levels of internalised receptor found in GFP-Rab7 late endosomes expressed in HEK293 cells.

In agreement with this, biochemical analysis revealed that the intracellular loop of the γ2 subunit is a substrate for ubiquitination, and this ubiquitination is severely reduced when the 7 lysines within the N-terminal end of the intracellular loop are mutated. These results suggest that like some non-neuronal membrane proteins, including the EGF receptor (Levkowitz et al., 1998), interleukin-2 receptor (Rocca et al., 2001), CXCR4 receptor (Marchese and Benovic, 2001), β2 adrenergic receptor (Shenoy et al., 2001), growth hormone receptor (VanKerkhof and Strous, 2001) and the LDL receptor related protein (Melman et al., 2002), $GABA_A$ receptor degradation within the endocytic pathway is dependent on the ubiquitination of lysine residues within the intracellular domain of the γ2 subunit. Importantly, these lysines were not found to be necessary for the internalisation of C1 or heteromeric αβγ $GABA_A$ receptors, suggesting that ubiquitin plays an exclusive role in the membrane sorting but not internalisation of $GABA_A$ receptors. Moreover, these results highlight an important role for ubiquitin in $GABA_A$ receptor degradation outside the proteasome.

In addition to a lysine stretch in the intracellular loop of the γ2 subunit, the role of S327 in $GABA_A$ receptor post endocytic sorting was also investigated. Antibody labeling experiments were carried out in HEK293 cells expressing GFP-Rab7 and $C1^{S327A}$ or $\alpha1\beta3^{9E10}\gamma2L^{S327A}$. Interestingly, quantitative analysis of the colocalisation levels of internalised receptors with GFP-Rab7 positive endosomes revealed that similar to the results obtained with mutation of the lysine stretch, late endosomal/lysosomal targeting was significantly reduced when S327 was mutated.

The ubiquitination of proteins occurs in three steps mediated by three different enzymes: E1 (activation), E2 (conjugation) and E3 (ligation). Substrate recognition is carried out by E3 type enzymes, which in some cases recognize their targets by binding to phosphorylated or dephosphorylated residues (for review see Pickart 2001). S327 is a known substrate for PKC phosphorylation (Brandon et al., 2002; Song and Messing, 2005), and work presented here suggests that mutation of this site reduces the late endosomal/lysosomal targeting of $GABA_A$ receptors in a

similar manner to the mutation of a lysine stretch. Therefore, a possible role for S327 in the ubiquitination of the intracellular loop of the $GABA_A$ receptor γ2 subunit was investigated. Interestingly, γ2 subunit ubiquitination was found to be completely abolished by a single point mutation at S327. These results indicate that S327 can regulate the ubiquitination of the γ2 subunit and hence the late endosomal/lysosomal targeting of $GABA_A$ receptors.

The role of S327 phosphorylation, however, is not fully understood. Evidence in heterologous expression systems is contradictory, possibly due to the existence of multiple PKC isoforms (Song and Messing, 2005). Whereas phosphorylation of S327 by phorbol ester induced PKC activation has been shown to downregulate $GABA_A$ receptor function (Kellenberger et al., 1992; Krishek et al., 1994), phosphorylation of the same site using the catalytically active domain of PKC (PKM) results in an enhancement of $GABA_A$ receptor expression (Lin et al., 1996). In hippocampal neurons, evidence suggests that dephosphorylation at this site by calcineurin activation results in a downregulation of $GABA_A$ receptor expression at the cell surface (Wang et al., 2003). In addition, activation of PKC by phorbol esters results in a downregulation of $GABA_A$ receptor cell surface expression in a manner that is independent of receptor phosphorylation (Connolly et al., 1999). Given the complications of PKC activation in heterologous expression systems, it is unclear, whether in the endocytic pathway S327 mediates late endosomal/ lysosomal sorting in its phosphorylated or dephosphorylated state.

The above results identified ubiquitination as a molecular signal for the degradation in the lysosome of γ2 containing $GABA_A$ receptors. Moreover, S327 was found to be necessary for inducing ubiquitination of the γ2 subunit, raising the possibility that this process may be regulated by phosphorylation. Together these results revealed a critical role for receptor post-translational modification by both ubiquitination and possibly phosphorylation in the γ2 subunit dependent endocytic sorting of $GABA_A$ receptors.

5. Functional significance of modulating $GABA_A$ receptor endocytic sorting

Introduction

In the previous chapters, the endocytic sorting fate of $GABA_A$ receptors was investigated revealing that $GABA_A$ receptors are targeted to late endosomes/ lysosomes via a mechanism dependent on the γ2 subunit. Using β3/γ2 chimeras coupled with site directed mutagenesis it was evident that the late endosomal/ lysosomal targeting of $GABA_A$ receptors was dependent on the ubiquitination of a lysine stretch within the intracellular loop of the γ2 subunit. Furthermore the ubiqutination of these lysines was shown to be dependent on the presence of S327 a known phosphorylation site (Kellenberger et al., 1992; Krishek et al., 1994). This indicated the possible existence of a mechanism to control $GABA_A$ receptor ubiquitination dependent upon the phosphorylation state of S327.

Synapses are able to modify in structure and function in response to stimuli. Changes in the efficacy of synapses are important for modulating the input-output relationship of neurons, and this plasticity is thought to underlie higher cognitive functions such as learning and memory as well as playing a key role in during development (Gaiarsa et al., 2002; Kittler and Moss, 2003; Esteban, 2003; Collingridge et al., 2004). The efficacy of synaptic transmission can be modulated by presynaptic changes and postsynaptic changes. Research into receptor function has revealed two main mechanisms for regulating synaptic efficacy: changing receptor channel properties with post-translational modification, or regulating the number of receptors accumulated at synapses (Kittler and Moss, 2003; Esteban, 2003; Collingridge et al., 2004).

In keeping with this, both excitatory and inhbitory receptors have been shown to be highly mobile, capable of altering their number at synapses in response to various forms of stimuli (Kittler et al., 2003; Collingridge et al., 2004). Three distinct mechanisms have been shown to play critical roles in changing the number of receptors at synaptic sites thereby regulating synaptic efficacy.

A number of studies have described the ability of AMPA, glycine and $GABA_A$ receptors to dynamically move in and out of synaptic sites by lateral diffusion

accross the neuronal membrane. Studies on the lateral mobility of receptors including AMPA, glycine, and $GABA_A$ receptors have revealed that there are significant rates of exchange between synaptic and extrasynaptic pools of these ligand-gated ion channels (Choquet and Triller, 2003; Dahan et al., 2003; Tardin et al., 2003, Thomas et al., 2005, Jacob et al., 2005) and this lateral diffusion has been shown to be directly regulated under conditions that result in receptor downregulation (Tardin et al., 2003).

In addition, the regulation of endocytosis has been established as being a potent mechanism for altering the number of receptors found at the cell surface together with the efficacy synaptic transmission (Collingridge et al., 2004). A number of neuronal receptors, including, AMPA, NMDA, Kainate, Glycine and $GABA_A$ receptors, have been shown to undergo clathrin mediated endocytosis either constitutively or in response to stimuli (Collingridge et al., 2004; Kittler and Moss, 2003). For $GABA_A$ receptors, endocytosis has been shown to occur constitutively in cultured neurons, and this has been shown to be inhibited by conditions that block clathrin mediated endocytosis. Furthermore, blockade of $GABA_A$ receptor internalisation has been shown to cause an upregulation of GABAergic function further confirming the role of receptor trafficking as a mechanism for altering the strength of inhibitory synapses. Importantly, $GABA_A$ receptor internalisation has recently been shown to be a dynamically regulated process. $GABA_A$ receptor endocytosis has been recently been shown to be regulated in a negative fashion by receptor phosphorylation and this mechanism has been shown to be of importance in the downregulation of $GABA_A$ receptor function in response to D3 receptor activation in the nucleus accumbens (Kittler et al., 2005; Chen et al., 2006).

In addition to regulating the lateral diffusion and internalisation of synaptic receptors, recent evidence has implicated endocytic sorting as a method for dynamically altering receptor cell surface expression. For example, AMPA receptor recycling has recently been described as a critical mechanism for altering synaptic strength under physiological conditions such as LTP where there is an increase in the number of AMPA receptors at the cell surface, which originate from a recycling

pool of receptors (Park et al., 2004). The endocytic sorting of $GABA_A$ receptors has also recently been highlighted as a potential mechanism for regulating synaptic inhibition. The β subunits of $GABA_A$ receptors have been shown to bind HAP1, and overexpression of this protein in hippocampal cells resulted in an increase in GABAergic currents compared to mock transfected cells. Furthermore, this increase was correlated by an increase in cell surface receptors as demonstrated by biotinylation experiments (Kittler et al., 2004b). Interestingly, internalised $GABA_A$ receptors have been associated with an intracellular pool close to the post-synaptic density, suggesting the possible existence of a reserve pool for dynamically increasing the number of receptors at the cell surface (vin Rijnsoever et al., 2005).

Having established the molecular determinants for $GABA_A$ receptor endocytic sorting this chapter focuses on analysing the possible functional consequences of regulating this process with particular attention to changes in the cell surface expression levels of $GABA_A$ receptors and their accumulation at post-synaptic sites.

Results

It is evident from the data presented in the preceding chapters that the late endosomal/lysosomal targeting of $GABA_A$ receptors is largely determined by the presence of the γ2 subunit. Leupeptin is a generic lysosomal protease inhibitor that has previously been used to block lysosomal degradation of internalised neuronal receptors (Ehlers, 2000; Fairfax et al., 2004; Ju et al., 2004; Kittler et al., 2004b). Therefore, to address if modifying the lysosomal degradation of $GABA_A$ receptors can alter their accumulation on the plasma membrane, the effects of leupeptin on $GABA_A$ receptor function were assessed using electrophysiological, biochemical and immuncytochemical approaches.

Leupeptin treatment enhances the functional expression of recombinant $GABA_A$ receptors in HEK293 cells

The effects of leupeptin on $GABA_A$ receptor function were assessed in transfected HEK293 cells expressing receptors of distinct subunit combinations. These experiments used patch clamp recordings in the whole cell mode to measure the magnitude of GABA induced currents (I_{GABA}) over time in the presence of non-saturating concentrations of agonist (Wooltorton et al., 1997; Connolly et al., 1999a).

For HEK293 cells expressing $GABA_A$ receptors composed of α1β3 subunits, I_{GABA} induced by the rapid application of a non-saturating concentration of agonist (5μM GABA, Woolworton et al., 1997, Connolly et al., 1999a), were well maintained over a time course of 40min at 32-33°C (Fig. 5.1). The introduction of leupeptin via intracellular dialysis had little effect on I_{GABA} of receptors composed of α1β3 subunits over the same recording period. Cells expressing $GABA_A$ receptors composed of α1β3γ2L subunits showed similar well maintained currents under control conditions over a 40min time period. However, in contrast to cells expressing receptors composed of α1β3 subunits, leupeptin produced a significant time dependent increase in the magnitude of I_{GABA} for cells expressing α1β3γ2L

subunits. After 30min leupeptin caused a 102.3% ± 21 increase in I_{GABA} compared to that observed after 1 min for $\alpha1\beta3\gamma2L$ containing $GABA_A$ receptors (Fig. 5.1). Together these results suggest that blocking lysosomal degradation specifically modulates the functional expression of $GABA_A$ receptors composed of $\alpha1\beta3\gamma2L$ subunits, which is consistent with the cell biological experiments presented in chapter 3 which illustrate a critical role for the $\gamma2$ subunit in regulating the delivery of $GABA_A$ receptors to late endosomal/lysosomal structures. Moreover, given that both $GABA_A$ receptors composed of $\alpha1\beta3$ or $\alpha1\beta3\gamma2L$ subunits can undergo constitutive endocytosis (Fig. 3.1) these results suggest that leupeptin treatment can alter GABAergic function without affecting internalisation. Most likely, the blockade of receptor degradation in the lysosome causes an accumulation of receptors along the endocytic pathway resulting in a reversal of the sorting decision at the level of the early endosome (Fig. 5.2)

Studies in chapter 4 also identified specific amino acid motifs within the $\gamma2$ subunit that regulate $GABA_A$ receptor delivery to late endosomes. Therefore, the effects of leupeptin on the function of wild typre receptors composed of $\alpha1\beta3\gamma2L$ were compared to those composed of $\alpha1\beta3\gamma2L^{K7R}$, and $\alpha1\beta3\gamma2L^{S327A}$ subunit combinations. Interestingly, leupeptin treatment resulted in a small albeit significant ($P<0.05$) increase in evoked GABAergic currents from HEK293 cells expressing receptors composed of $\alpha1\beta3\gamma2L^{K7R}$ or $\alpha1\beta3\gamma2L^{S327A}$ subunits (Fig. 5.3). However, this increase was significantly lower than that obtained with cells expressing $\alpha1\beta3\gamma2L$ subunits, suggesting that mutation of the lysine stretch or S327 causes a reduction in the downregulation of $GABA_A$ receptors in the lysosome.

The results presented above, highlight the importance of endocytic sorting in regulating the number of receptors at the cell surface. Moreover the data using the $\gamma2L$ mutants ($\gamma2L^{K7R}$ or $\gamma2L^{S327A}$) and leupeptin correlates with the findings that

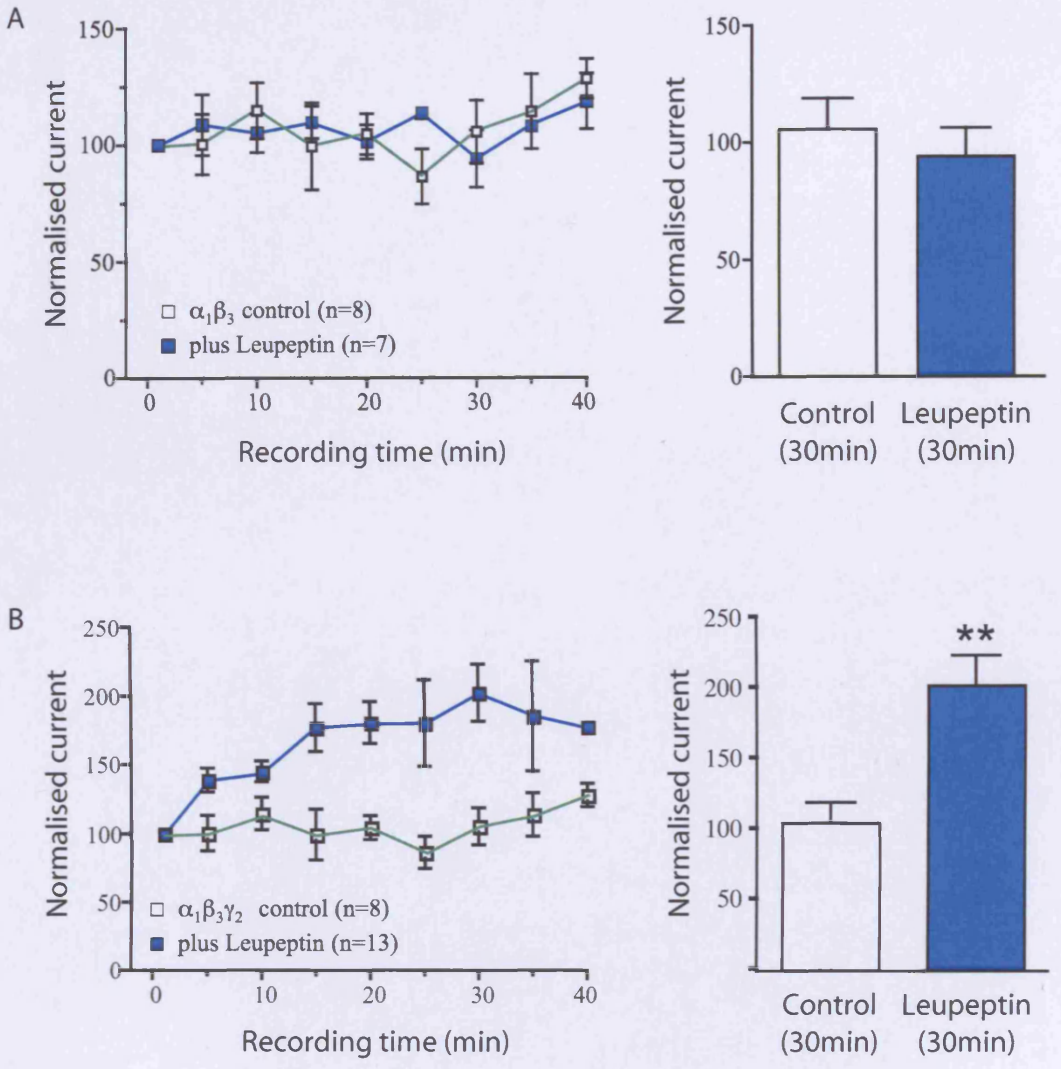


Figure 5.1: Analysis of GABA evoked currents in HEK293 cells expressing α1β3 or α1β3γ2L in the presence or absence of leupeptin. (A) HEK293 cells expressing α1 and β3 subunits were able to produce stable currents when activated with GABA (5μM). In the presence of leupeptin (40μM perfused through the patch pipette) there was no significant change in the normalised current compared to the control. (B) Evoked GABAergic currents in HEK293 cells expressing α1, β3, and γ2L subunits were significantly increased in the presence of leupeptin over time showing a 102.3% ± 21 increase in amplitude after 30min. (* $P<0.05$, T-test)

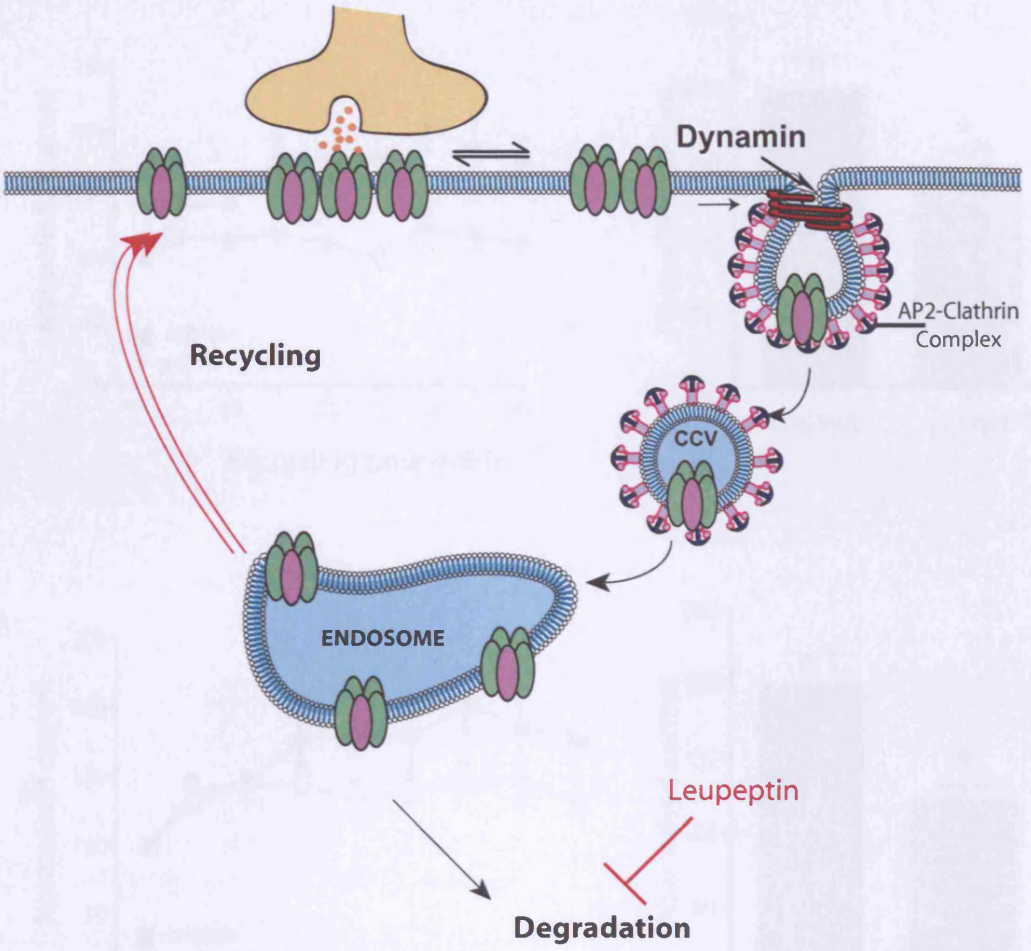


Figure 5.2: Effect of leupeptin on $GABA_A$ receptor endocytosis. The inhibition of $GABA_A$ receptor degradation can change the sorting decision in the early endosome and increase the levels of receptor recycling (red).

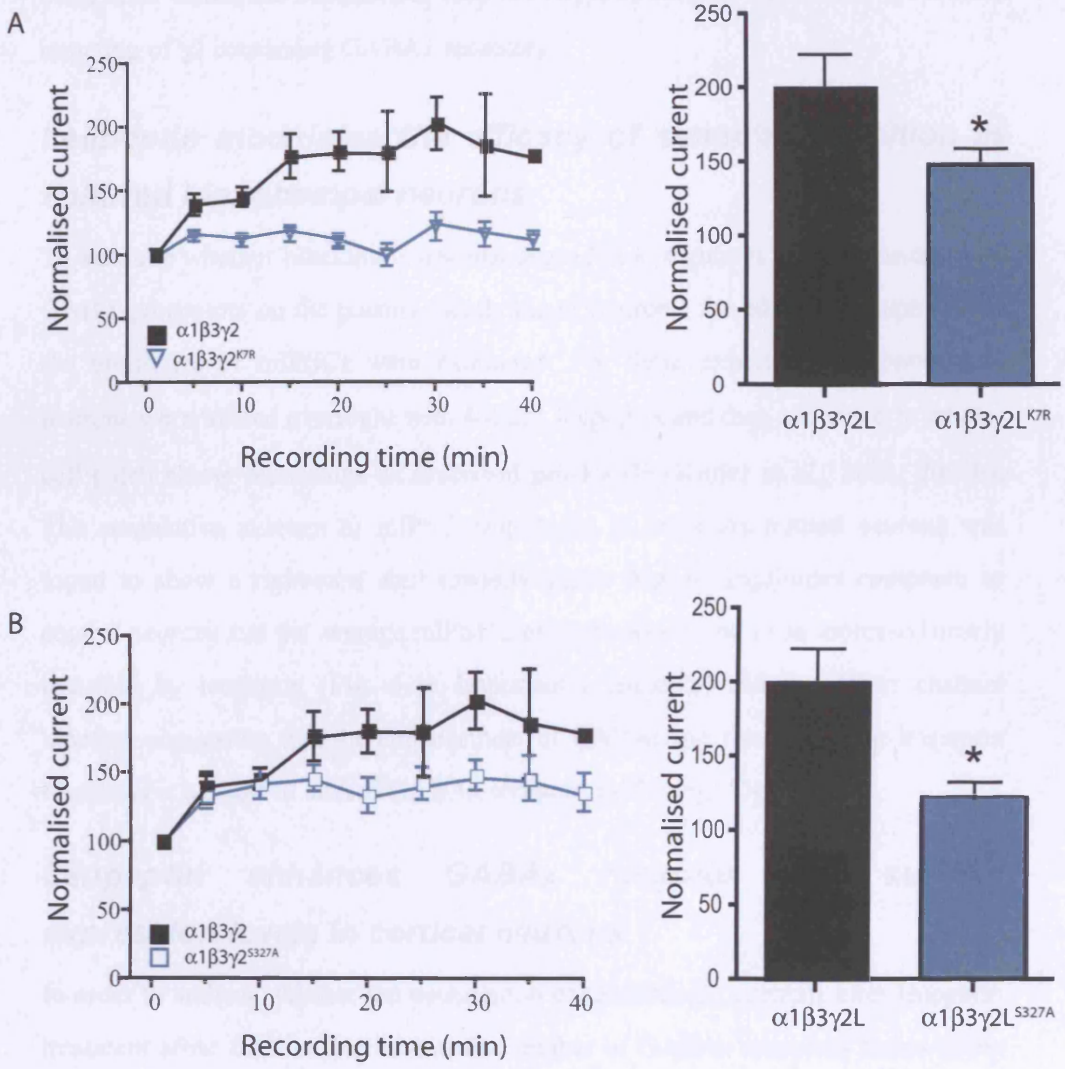


Figure 5.3: Analysis of GABA evoked currents in HEK293 cells expressing α1β3γ2L α1β3γ2L^{K7R} or α1β3γ2L^{S327A} in the presence or absence of leupeptin. (A) Evoked GABAergic currents in HEK293 cells expressing α1, β3, and γ2L subunits (black squares) were significantly increased in the presence of leupeptin over time showing a 102.3% ± 21 increase in amplitude after 30min. In contrast, the normalised current at t=30min was significantly lower in leupeptin treated HEK293 cells expressing α1β3γ2L^{K7R} (grey triangles) or (B) α1β3γ2L^{S327A} (grey open squares). (* P<0.05, T-test)

these sites within the intracellular loop are required for late endosomal/ lysosomal targeting of γ2 containing $GABA_A$ receptors.

Leupeptin modulates the efficacy of synaptic inhibition in cultured hippocampal neurons

To examine whether blocking lysosomal degradation regulates the accumulation of $GABA_A$ receptors on the plasma membrane of neurons, the effects of leupeptin on the properties of mIPSCs were examined. For these experiments hippocampal neurons were treated overnight with 400μM leupeptin and then subjected to whole-cell patch clamp recordings as described previously (Kittler et al., 2001; 2004b). The cumulative average of mIPSC amplitudes in leupeptin-treated neurons was found to show a rightward shift towards higher higher amplitudes compared to control neurons and the average mIPSC amplitude was found to be increased nearly two-fold by leupeptin (Fig. 5.4). Importantly leupeptin did not affect channel kinetics, suggesting that the enhancement of GABAergic function upon leupeptin treatment is a result of altered $GABA_A$ receptor trafficking (Fig. 5.4).

Leupeptin enhances $GABA_A$ receptor cell surface expression levels in cortical neurons

In order to address whether the potentiation of GABAergic currents after leupeptin treatment arose from an increase in the number of $GABA_A$ receptors found at the cell surface, biotinylations were carried out in 15DIV cultured cortical neurons treated overnight with 400μM leupeptin. Surface proteins were labeled with biotin under non-permeabilising conditions. After lysis in RIPA buffer, biotinylated proteins were precipitated with neutravidin beads. Precipitates were resolved by SDS electrophoresis and immunoblotted against the $GABA_A$ receptor β3 subunit. Although cortical neurons express a range of $GABA_A$ receptor α, β and γ subunits, the β3 subunit is expressed at significantly higher levels than the other β subunits, therefore blotting for this subunit should reliably detect most subtypes expressed in these cells (Kittler et al., 2004b). Biotinylated $GABA_A$ receptors were detected

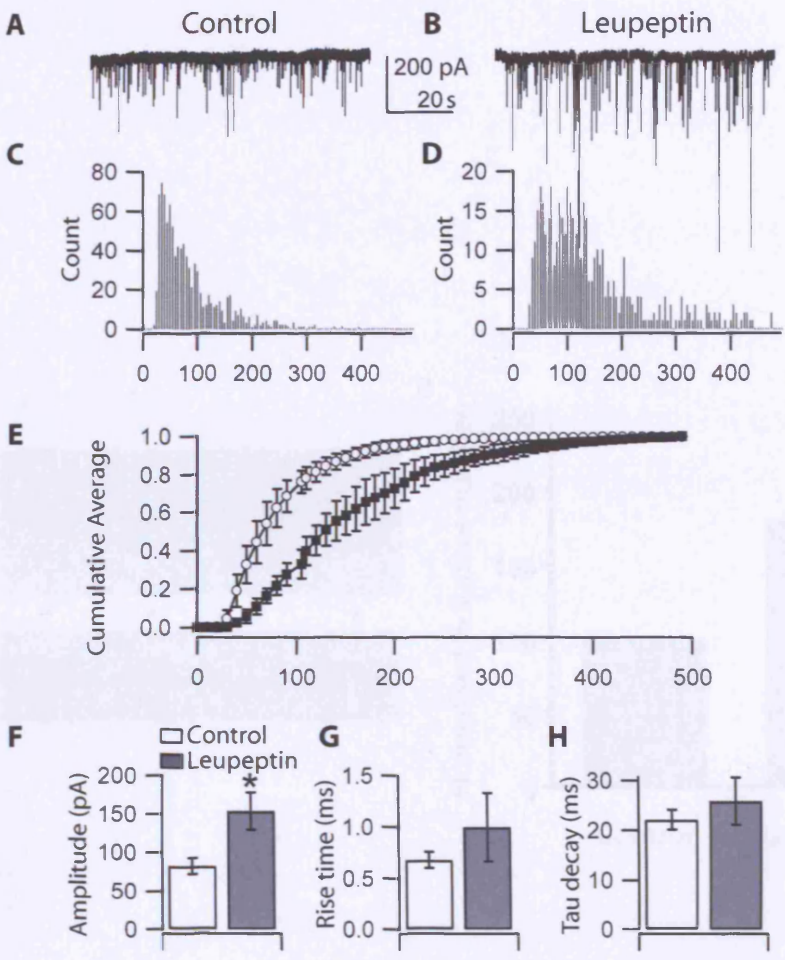


Figure 5.4: Analysis of mIPSCs in hippocampal neurons with leupeptin treatment. Whole-cell patch clamp recordings (A) and mIPSC histograms (C) from control neurons and neurons treated with leupeptin (B and D). The cumulative average of mIPSC amplitudes in leupeptin-treated neurons (filled symbols) is shifted to higher amplitudes compared to control neurons (open symbols) (E) and the average mIPSC amplitude is increased nearly two-fold by leupeptin (F). While leupeptin does not affect the time constant of decay (H), the mIPSC rise time (10-90%) is slower in leupeptin-treated neurons (G). (* $P<0.05$, T-test).

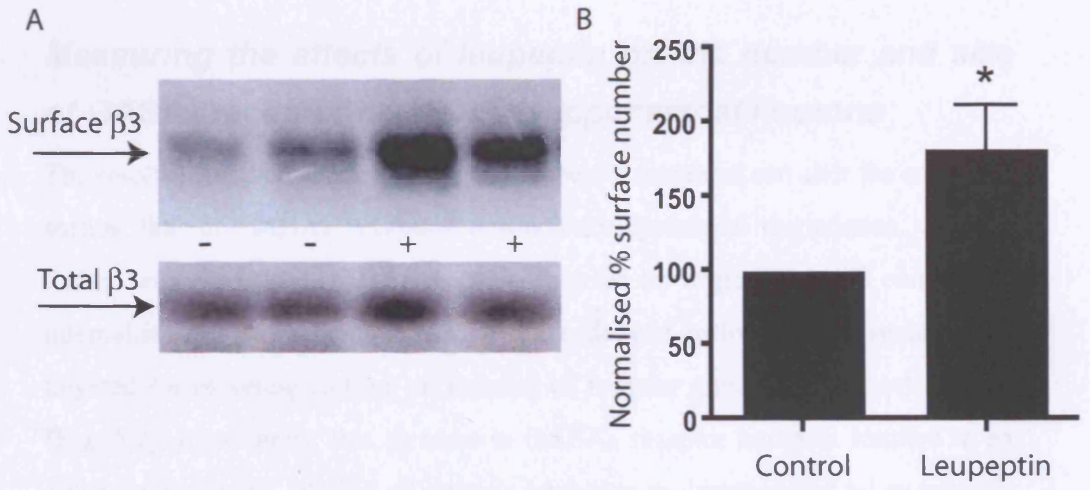


Figure 5.5: Leupeptin treatment results in an increase in $GABA_A$ receptors expressed at the cell surface. (A) Control untreated (-) cortical neurones, or neurones treated overnight with 400μM leupeptin (+) were biotinylated. Cells were then lysed and cell surface biotinylated receptor populations were purified with neutravidin immobilised on beads, separated by SDS-PAGE followed by quantitative immunoblotting with a rabbit polyclonal antibody to the $GABA_A$ receptor β3 subunit. (B) Leupeptin treatment resulted in an 82.4% ± 30 increase in the levels of $GABA_A$ receptor expressed at the cell surface of cultured cortical neurons. (* $P<0.05$, T-test; n=5)

using $^{[125]}I$ coupled secondary antibody, and levels were quantified with a phosphorimager. Bands for the β3 subunit were normalised to total protein as determined by a BCA protein assay and control levels of biotinylated β3 were assigned 100%. Interestingly, leupeptin treatment resulted in a signficant (82.4% ± 30, $P<0.05$) increase in the cell surface expression of the β3 subunit (Fig. 5.5). These results indicate that the increase in GABAergic function caused by leupeptin treatment is due to an increase in the cell surface expression of $GABA_A$ receptors.

Measuring the effects of leupeptin on the number and size of $GABA_A$ receptor clusters in hippocampal neurons

The results presented above suggest that leupeptin treatment can alter the endocytic sorting fate of $GABA_A$ receptors destined for lysosomal degradation. In cells treated with leupeptin, $GABA_A$ receptors targeted for degradation will continue to internalise but will accumulate along the endocytic pathway and eventually be targeted for recycling causing an increase of receptor numbers at the cell surface (Fig. 5.2). Importantly, this increase in $GABA_A$ receptor numbers resulted in an enhancement in the efficacy of synaptic inhibition as demonstrated by an increase in the amplitude of mIPSCs suggesting an increase in receptor number at inhibitory sites. To provide further evidence for an increase in receptor number at inhibitory synapses in response to leupeptin treatment, quantitative immunofluorescence techniques were utilised to measure the number of $GABA_A$ receptor clusters in control neurons and those treated with leupeptin.

Leupeptin treatment results in an increase in $GABA_A$ receptor cluster number in both distal and proximal dendrites

12-16DIV hippocamapal neurons treated overnight with 400μM leupeptin were fixed in 4% PFA. Cell surface $GABA_A$ receptor clusters were identified by staining for the γ2 subunit under non-permeabilising conditions. After permeabilisation, anti-VIAAT was used for synaptic identification and MAP2 for neuronal and dendritic identification.

Detailed analysis of cluster size and number was carried out in the distal and proximal sections of neuronal dendrites in cells treated with leupeptin overnight. The cluster size (in μm^2) and number, independent of synaptic colocalisation, was recorded for each 25μm section of neuronal process. Analysis of the total area occupied by clusters in leupeptin treated cells revealed a 43.65%± 17.66 increase in the distal dendrites (Fig. 5.6) and a 36.81%± 9.81 increase in the proximal dendrites (Fig. 5.7) when compared to control numbers. The number of clusters were found to significantly increase by 46.1% ± 17 in distal dendrites (Fig. 5.6) and 45.7% ± 14 in proximal dendrites (Fig. 5.7). Interestingly, the size distribution of clusters revealed that this increase affected all clusters, as there was no change in the distibution curves (Figs. 5.6 & 5.7).

These results strongly suggest that leupeptin treatment results in the formation of new clusters.

Leupeptin treatment results in an increase in $GABA_A$ receptor cluster size in the AIS

The axon initial segment (AIS) is a major site for GABAergic innervation (Christie and De Blas, 2003). In addition, altered $GABA_A$ receptor accumulation at this cellular specialisation has been strongly implicated in the pathology of schizophrenia (Volk et al., 2002). In order to investigate the possible effects of modulating receptor endocytic sorting on $GABA_A$ receptor accumulation at subcellular specialisations, high resolution image analysis of $GABA_A$ receptor clusters was carried out in the AIS after leupeptin treatment

Similar to the results obtained in the dendrites, leupeptin treatment caused a 45.6% ± 15 increase in the total area occupied by clusters compared to clusters in control cells. Intriguingly, the determinant for this increase differed from that obtained in the dendritic analysis. Whereas in both distal and proximal dendrites there was a significant increase in the total number of clusters, the AIS cluster number was not significantly increased. Instead, there was a significant increase in the size of the $GABA_A$ receptor clusters as observed by a rightward shift in the size distribution

trace (Fig. 5.7). Interestingly, these results indicate that perhaps there are structural restrictions (quite possibly related to the network of cytoskeletal proteins that allow membrane protein anchoring in the AIS (Winckler et al., 1999)) within the AIS that prevent a regulation of receptor cluster number.

Leupeptin does not alter the ratio of synaptic/non-synaptic γ2 containing $GABA_A$ receptor clusters

High resolution images of distal and proximal dendrites were taken and analysed using Metamorph imaging software. The number of γ2 subunit clusters present in a 25μm length of dendrite was recorded. Clusters found overlapping or opposing VIAAT clusters were considered synaptic, whereas all other clusters were considered non-synaptic.

The ratio of non-synaptic to synaptic γ2 containing $GABA_A$ receptor clusters was calculated for both cells treated with leupeptin and those untreated. Results revealed that 90.7 %± 4 of $GABA_A$ receptor clusters are synaptic in untreated cells (Fig. 5.9). Similarly, in cells treated overnight with leupeptin 91.2%± 3 of clusters were found to be synaptic (Fig. 5.9). These results were not significantly different from each other suggesting that leupeptin increases the number of $GABA_A$ receptor clusters at the cell surface without affecting their ability to form synaptic connections.

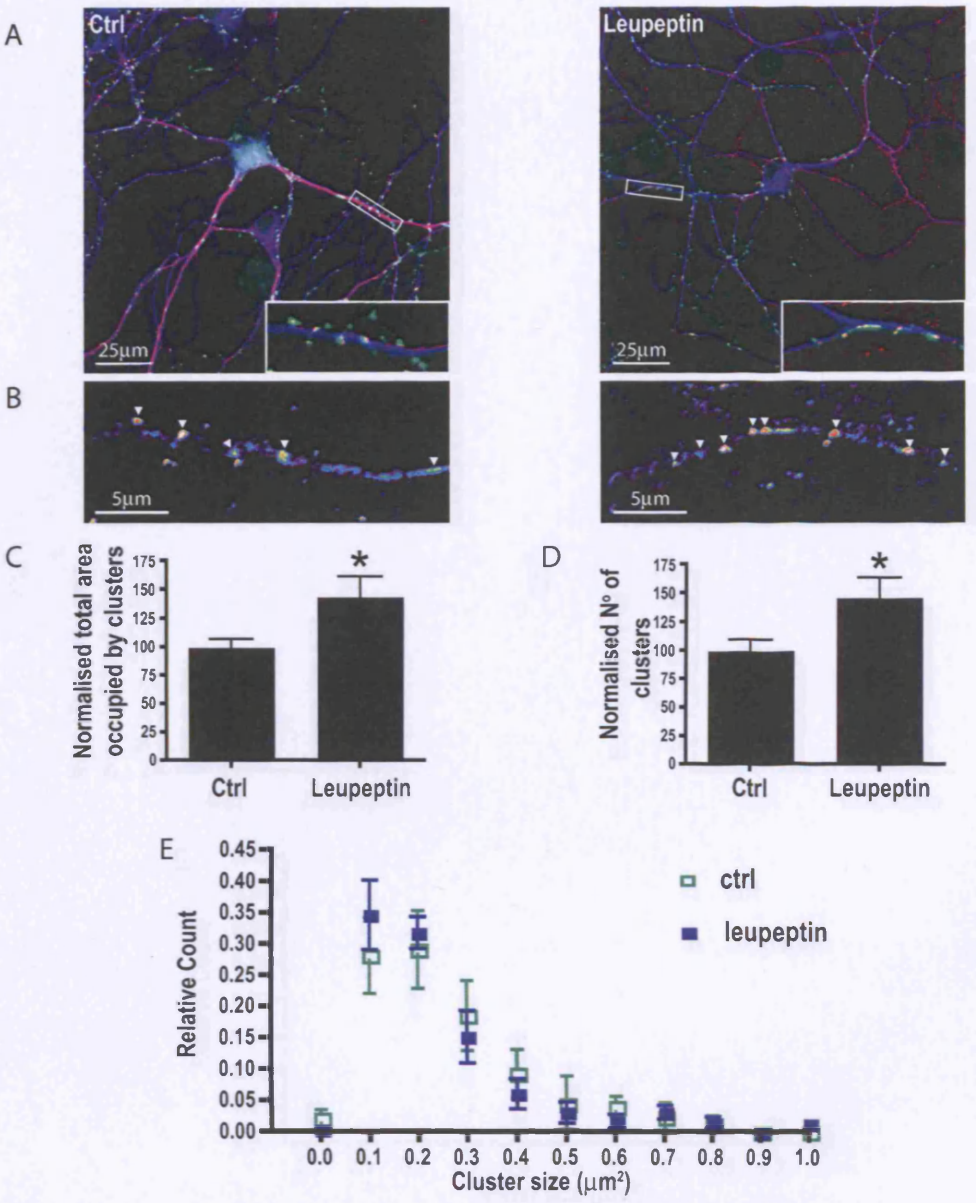


Figure 5.6: Distribution analysis of cluster size and number in the distal dendrites. Cell surface of labeling $GABA_A$ receptors was carried out in 12-16DIV cultured hippocampal neurons treated overnight with 400µM leupeptin. (A) $GABA_A$ receptor clusters were identified using a guinea pig antibody to the N-terminus of the γ2 subunit (red). Presynaptic inhibitory terminals were labeled with anti-VIAAT (green) and neuronal dendrites were stained for using anti-MAP2 (blue). High resolution confocal images were taken of the distal dendrites (35µm away from the cell body). (B) $GABA_A$ receptor clusters were identified by an increase in intensity around a central point (arrow heads). The size and number of clusters was recorded for each dendrite and results were normalised to the control cells. Leupeptin treatment caused a significant increase in the total area occupied by clusters (C) and the number of clusters (D). The relative size distribution of clusters shows no significant change between both sets of data. (* $P<0.05$, T-test; n=9)

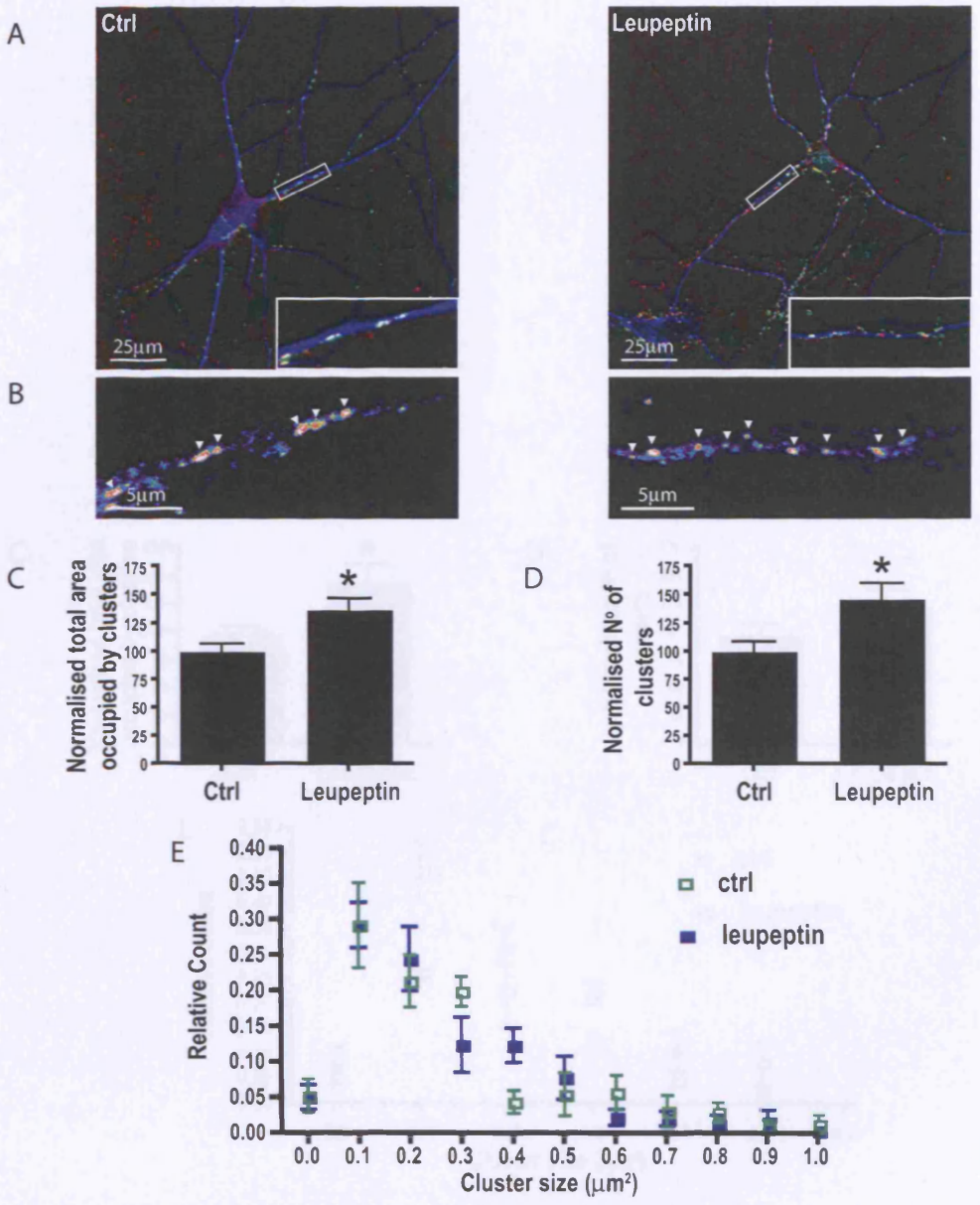


Figure 5.7: Distribution analysis of cluster size and number in the proximal dendrites. Cell surface labeling of $GABA_A$ receptors was carried out in 12-16DIV cultured hippocampal neurons treated overnight with 400µM leupeptin. (A) $GABA_A$ receptor clusters were identified using a guinea pig antibody to the N-terminus of the γ2 subunit (red). Presynaptic inhibitory terminals were labeled with anti-VIAAT (green) and neuronal dendrites were stained for using anti-MAP2 (blue). High resolution confocal images were taken of the proximal dendrites. (B) $GABA_A$ receptor clusters were identified by an increase in intensity around a central point (arrow heads). The size and number of clusters was recorded for each dendrite and results were normalised to the control cells. Leupeptin treatment caused a significant increase in the total area occupied by clusters (C) and in the number of clusters (D). The relative size distribution of clusters is equal for both control and leupeptin treated clusters. (* $P<0.05$, T-test; n=9)

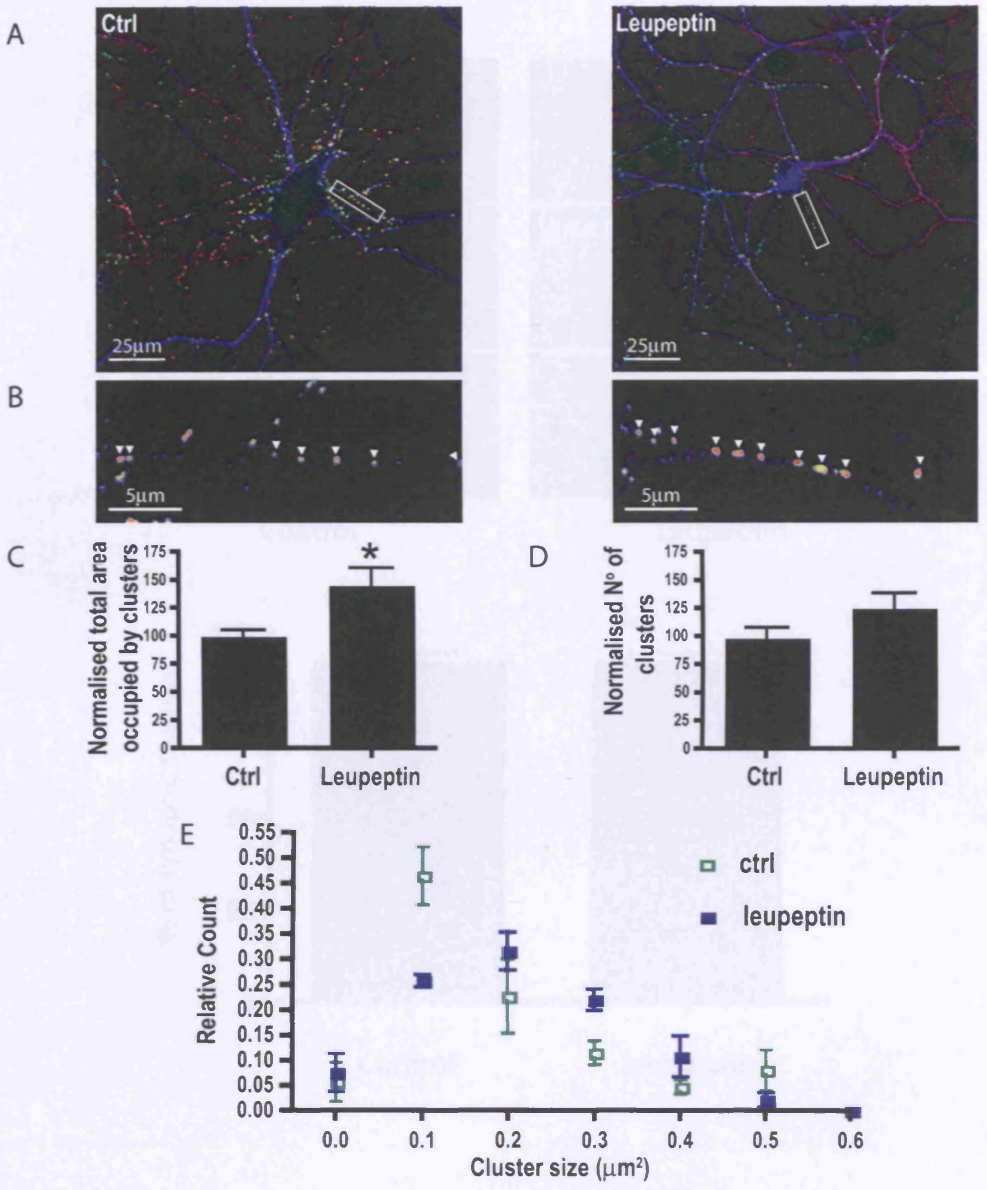


Figure 5.8: Distribution analysis of cluster size and number in the AIS. Cell surface labeling of $GABA_A$ receptors was carried out in 12-16DIV cultured hippocampal neurons treated overnight with 400µM leupeptin. (A) $GABA_A$ receptor clusters were identified using a guinea pig antibody to the N-terminus of the γ2 subunit (red). Presynaptic inhibitory terminals were labeled with anti-VIAAT (green) and neuronal dendrites were stained for using anti-MAP2 (blue). High resolution confocal images were taken of the AIS (defined by MAP2 negative staining). (B) $GABA_A$ receptor clusters were identified by an increase in intensity around a central point (arrow heads). The size and number of clusters was recorded for each AIS and results were normalised to the control cells. Leupeptin treatment caused a significant increase in the total area occupied by clusters (C) but not in the number of clusters (D). The relative size distribution of clusters shows a rightward shift indicative of an increase in the number of larger clusters. (* $P<0.05$, T-test; n=9)

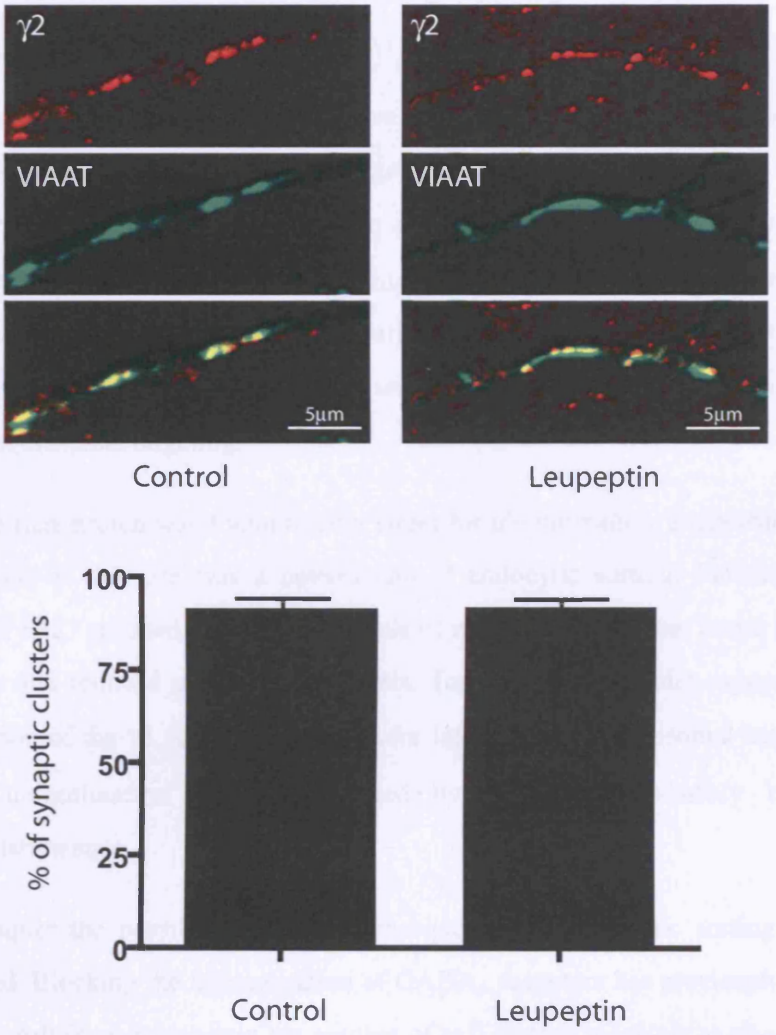


Figure 5.9: Leupeptin does not affect the ability of clusters ot form synaptic connections. The percentage of clusters colocalising or opposing presynaptic inhibitory terminals was calculated in control cells and cells treated overnight with 400µM leupeptin. 90.7% ± 4 and 91.2% ± 3 of clusters were found to be synaptic in control and leupeptin treated cells respectively (n=9).

Discussion

In this study $GABA_A$ receptors are shown to be internalised to two endocytic sorting fates. The sorting decision is largely regulated by subunit composition where the γ2 subunit plays a critical role in allowing for late endosomal/lysosomal targeting. Using a chimeric system and site directed mutagenesis together with antibody labeling experiments and quantitative confocal microscopy a lysine rich stretch, and a known phosphorylation site were identified as key regulators of late endosomal/lysosomal targeting.

This lysine rich stretch was found to be a target for ubiquitination, suggesting that ubiquitination at this site was a determinant of endocytic sorting. Furthermore, mutation of S327 resulted in decreased levels of internalised receptor found in late endosomes and reduced ubiquitination levels. Together, these results suggest that ubiquitination of the γ2 subunit is required for late endosomal/lysosomal targeting and this ubiquitination can be regulated by S327 quite possibly by its phosphorylation state.

In this chapter the possible functional consequences of endocytic sorting were investigated. Blocking the internalisation of $GABA_A$ receptors has previously been shown to result in an increase in the number of cell surface receptors as shown by electrophysiological recordings and cell surface biotinylations (Kittler et al., 2000; vin Rijnsoever et al., 2005). In addition, overexpression of HAP1 a modulator of endocytic sorting of $GABA_A$ receptors by its interaction with the β subunits, can also cause an increase in cell surface receptor number (Kittler et al., 2004b). $GABA_A$ receptor degradation in the lysosome has been previously shown to be blocked by the protease inhibitor leupeptin (Kittler et al., 2004b). Here, leupeptin was used to block the degradation of $GABA_A$ receptors targeted to the late endosomes/lysosomes and change the sorting fate of internalised receptors. The functional consequences of changing this sorting decision was then investigated on inhibitory currents, surface receptor number as well as $GABA_A$ receptor cluster size and number.

Electrophysiological recordings of HEK293 cells expressing recombinant $GABA_A$ receptors consisting of $\alpha1\beta3$ or $\alpha1\beta3\gamma2L$ revealed that whereas $\alpha1\beta3$ expressing cells were able to produce stable currents that were unaffected by leupeptin addition, cells expressing $\alpha1\beta3\gamma2L$ subunits showed a time dependent increase in the whole cell current upon acute leupeptin treatment. This approach confirmed a $\gamma2$ subunit dependence on the leupeptin effect suggesting that leupeptin can cause an enhancement of $GABA_A$ receptor functional expression, independent of receptor internalisation.

Importantly, HEK293 cells expressing $GABA_A$ receptors containing mutant forms of the $\gamma2$ subunit ($\gamma2^{K7R}$ or $\gamma2^{S327A}$) did not show such an extensive increase in the whole cell current when compared to cells expressing wild-type $\alpha1\beta3\gamma2L$ containing receptors confirming that these mutations cause a reduction in the late endosomal/lysosomal targeting of $GABA_A$ receptors.

Given that the $\gamma2L$ subunit is present in the majority of inhibitory synapses in the brain, the effect of leupeptin was also investigated in neuronal preparations. Cultured hippocampal neurons were treated overnight with leupeptin and mIPSCs were recorded. Similar to the results above, leupeptin caused an increase in the amplitude of mIPSCs without affecting channel kinetics. Furthermore, cell surface biotinylations on cortical neurons demonstrated that enhanced GABAergic function in response to leupeptin was a result of an increase in receptor number at the cell surface.

To examine where in the neurons these changes were occuring a detailed immunofluorescent study was carried out. The size and number of $\gamma2$ containing cell surface clusters was investigated in 13-16DIV hippocampal neurons and compared to those treated with leupeptin overnight. Leupeptin treatment resulted in a significant increase in the area occupied by clusters in all parts of the neuron examined, results that correlated with the previous findings of an increase in $GABA_A$ receptor numbers and an enhancement in GABAergic function. However,

whether this change was due to an increase in synaptic number or size varied in the different neuronal regions. In both the proximal and distal dendrites, leupeptin caused an increase in the number of clusters without affecting the size distribution. Importantly, the majority of clusters were found to be synaptic independent of leupeptin treatment suggesting that the new formation of clusters due to leupeptin treatment did not affect their ability to form synaptic connections.

The AIS is the site for action potential initiation (Matus, 2001). GABAergic innervation at this site is therefore of great importance in determining the decision on action potential propagation (Somogyi et al., 1983; Christie and de Blas, 2003). Furthermore, altered $GABA_A$ receptor expression in the AIS has been shown in schizophrenic patients further highlighting the importance of inhibition at this site (Blum and Mann, 2002; Benes and Berretta, 2001; Fritschy and Brunig, 2003). In cultured hippocampal cells treated overnight with leupeptin, there was and increase in the area of the neuron occupied by synaptic $GABA_A$ receptors. In the AIS, however, this change was achieved solely by a change in the size of GABAergic clusters. Importantly, an increase in synapse size in the AIS could result in a substantial influx of chloride concentrated in a relatively small area resulting in a large inhibitory response.

The data presented here therefore highlights the importance of regulating endocytic sorting in inhibitory synaptic transmission. In particular, it identifies a method by which only synaptic $GABA_A$ receptors can be dynamically regulated to enhance GABAergic function by allowing the formation of new synaptic connections, as well as an enlargement of existing ones.

6. Final Discussion

Summary

Recent evidence suggests that $GABA_A$ receptor trafficking is a dynamically regulated process (Kittler et al., 2001; Kittler and Moss, 2003; Collingridge et al., 2004, Kittler et al., 2005; Chen et al., 2006). This thesis describes studies carried out to gain further insight into the mechanisms that underlie control of the endocytic trafficking of synaptic $GABA_A$ receptors. To this end, molecular, biochemical and cell biological methodologies were used to analyse receptor trafficking in heterologous expression systems and cultured neurons.

The role of $GABA_A$ receptor subunit composition was investigated in HEK293 cells coexpressing recombinant $GABA_A$ receptor subunits and GFP-tagged endosomal markers. Receptors composed of $\alpha1\beta3$ or $\alpha1\beta3\gamma2L$ were found to constitutively internalise as previously shown (Connolly et al., 1999a,b; Kittler et al., 2000). However, the levels of colocalisation with the early endosomal marker GFP-2FYVE or the late endosomal/lysosomal marker GFP-Rab 7 were significantly different when comparing internalised $\alpha1\beta3$ and $\alpha1\beta3\gamma2L$. Whereas a small proportion of internalised $\alpha1\beta3$ receptors were found to be targeted to late endosomes for subsequent degradation in the lysosome, a significanlty larger proportion of $\alpha1\beta3\gamma2L$ receptors were found to be destined for lysosomal degradation in HEK293 cells. Although the levels of internalised receptors targeted for late endosomes in HEK293 cells overexpressing $GABA_A$ receptors are unlikely to occur in neurons, these results suggest a critical role for subunit composition in the targeting of $GABA_A$ receptors along the endocytic pathway as well as suggesting the possible existence of a regulatory mechanism acting on the $\gamma2$ subunit that can specfically alter the degradation of synaptic $GABA_A$ receptors.

Using a $\beta3/\gamma2$ chimera consisting of a $\beta3$ N-terminal backbone and the intracellular loop and TM4 of the $\gamma2$ subunit, it was evident that the $\gamma2$ subunit intracellular loop and TM4 were necessary and sufficient for this enhanced targeting to late endosomes in both HEK293 cells and cultured hippocampal neurons. A number of chimeras, consisting of amino acid replacements in the intracellular loop of the $\gamma2$

subunit with those of the β3 subunit, were created in order to map the possible molecular determinants within the γ2 intracellular loop or TM4 of the late endosomal/lysosomal targeting of γ2 subunit containing $GABA_A$ receptors. Interestingly, chimeras which lacked the fisrt 20 amino acids of the γ2 intracellular loop, were unable to reach late endosomal/lysosomal structures to the same level as the chimera which had the whole intracellular loop and TM4 of the γ2 subunit. These results strongly suggested that the late endosomal/lysosomal targeting of γ2 containing $GABA_A$ receptors was achieved by a molecular signal in the first 20 amino acids of the γ2 intracellular loop.

The 20 amino acid region, within the γ2 subunit intracellular loop, identified to be important in the late endosomal/lysosomal targeting of $GABA_A$ receptors contains many lysine residues which can be a target for covalent modification by the small polypeptide ubiquitin. The ubiquitination of proteins has been shown to be a key mechanism in regulating protein degradation in the proteasome as well as the lysosome. Importantly, a stretch of lysine residues within the intracellular loop of the $GABA_A$ receptor γ2 subunit was shown to be a target for ubiquitination. Furthermore, mutagenesis experiments revealed that these lysines were found to be necesasary for determining the late endosomal/lysosomal targeting of $GABA_A$ receptors. Importantly, this site was not necessary for receptor internalisation, suggesting that ubiquitination within this sorting signal might occur post-internalisation at the level of the early endosome. These findings are the first report to sugest that ubiquitination of $GABA_A$ receptors is important for the degradation of receptors outside the proteasome, within the endosomal pathway.

Phosphorylation of $GABA_A$ receptors has long been suggested as an important mechanism for regulating GABAergic function. Diverse studies on $GABA_A$ receptor phosphorylation have implicated this process in altering channel kinetics, open time, rate of desensitization and sensitivity to pharmacological agents (Brandon et al., 2002; Kittler et al., 2003; Song and Messing, 2005). Recently, $GABA_A$ recptor phosphorylation has also been shown to play a role in controlling

receptor cell surface stability. The binding of AP2 to a novel binding site within the β subunits of the $GABA_A$ receptor was shown to be regulated by the phosphorylation state of a conserved serine residue in all $GABA_A$ receptor β subunits (Kittler et al., 2005). Here, S327, a residue within the late endosomal/ lysosomal sorting signal in the intracellular loop of the γ2 subunit was shown to play a key role in determining the endocytic sorting of internalised $GABA_A$ receptors by regulating the ubiquitination of the γ2 subunit.

How S327 regulates the ubiquitination of $GABA_A$ receptors remains unclear. However, it is most likely that the phosphorylation state of this residues plays a major role. The ubiquitination of proteins requires 3 different enzymes (E1-3) which act at different stages of the ubiquitin pathway. Substrate recognition is carried out by E3 enzymes which bind their target protein and allow the ligation of the ubiquitin peptide to specific lysine residues. Due to their nature, E3 ligases are the most abundant of ubiquitin enzymes. Substrate recognition has been investigated for a number of E3 ligases, and although this varies between ligases, the phosphorylation state of residues among the target protein has been shown to be a recurrent mechanism for regulating substrate recognition. For example there are a number of E3 ligases that recognise their substrates where substrate recognition occurs by binding phosphorylated (Yaron et al., 1998; Skowyra et al., 1997; Feldman et al., 1997) or dephosphorylated (Honda and Yasuda, 1999) residues.

Intriguingly both phosphorylation and dephosphorylation of S327 on the γ2 subunit have been previously shown to cause a downregulation of $GABA_A$ receptors. In hippocampal neurons, evidence suggests that dephosphorylation at this site by NMDA receptor dependent activation of calcineurin results in a downregulation of $GABA_A$ receptor expression at the cell surface (Wang et al., 2003). Furthermore, the use of a constitutively active form of PKC (PKM) results in an enhancement of GABAergic function in transfected mouse fibroblasts in a manner that is partly dependent on phosphorylation at S327 (Lin et al., 1996). In contrast, the use of phorbol esters to activate PKC caused a time dependent downregulation of

GABAergic currents in cultured sympathetic neurons and heterologous expression systems. Interestingly, this effect was shown to be slightly reduced in heterologous expression systems when S327 was mutated (Kellenberger et al., 1992; Krishek et al., 1994). It has been speculated that this discrepancy in the results arises from the existence of multiple PKC isoforms that may produce different responses depending on their site of action (Song and Messing, 2005).

In this thesis, the effects of blocking receptor degradation at later stages of the endocytic pathway were investigated using leupeptin, a drug known to inhibit $GABA_A$ receptor degradation in the lysosome. Leupeptin treatment caused an enhancement of GABAergic function in a manner independent of receptor internalisation in both heterologous expression systems and cultured hippocampal neurons. Furthermore, biochemical analysis revealed that enhancement was due to an increase in cell surface receptor number. An increase in synaptic receptor number and synaptic inhibition can be achieved in 2 ways: an increase in the size of synapses, or an increase in the number of synaptic connections. Interestingly, immunocytochemical analysis of GABAergic synapses in cultured neurons revealed that leupeptin could affect both synapse size and number, although an increase in synapse size was achieved solely in the AIS, whereas in the dendrites the synapse size did not change, but there was an overall increase in the number of synapses. The reasons for this are unknown, although they suggest that perhaps in the AIS there are structural restrictions that do not allow for the formation of new synaptic connections.

$GABA_A$ receptor trafficking and synaptic plasticity

Activity dependent synaptic plasticity is thought to be important for certain forms of learning and memory, neuronal development and pathological states of neuronal excitability (Gaiarsa et al., 2002, Gaiarsa and Ben-Ari, 2006). Synaptic plasticity describes the ability of synapses to modify in structure and function in response to stimuli. The most characterised forms of plasticity, long-term potentiation (LTP) and long-term depression (LTD), describe the ability of a synapse to undergo long term changes in strength after repeated stimuli and is thought to be the basis for

learning and memory. In addition to LTP and LTD, another form of plasticity has been recently characterised. Whereas LTP and LTD describe the ability of indivudual synapses to change in response to stimuli, synaptic scaling allows for the regulation of the total synaptic strength of a neuron in response to activity by altering all synapses (Turrigiano et al., 1998; Turrigiano and Nelson, 2000).

Although synaptic plasticity is best characterised in glutamatergic synapses, these forms of plasticity have also been observed in inhibitory synapses. LTP and LTD of inhbitory synapses has been reported in various brain regions including: hippocampus (Caillard et al 1999a, Caillard et al 1999b, Stelzer et al 1987), cortex (Komatsu & Iwakiri 1993; Komatsu, 1996; Holmgren & Zilberter 2001) and cerebellum (Kano et al 1996, Kano et al 1992). In addition, synaptic scaling of inhibitory synapses has also been observed in cultured visual cortical pyramidal neurons (Kilman et al., 2002) and cultured hippocampal neurons (Stellwagen et al., 2005; Swanwick et al., 2006) and has been suggested to be a mechanism important in development and certain epilepsies (Kilman et al., 2002).

Changes in synaptic transmission can be achieved by modulating the efficacy of the postsynaptic response to transmitter release. This can be achieved by modifying receptor channel properties with post-translational modifications. Alternatively, under saturating levels of neurotransmitter, increasing or decreasing the number of receptors in the post-synaptic membrane would be expected to have a significant effect on synaptic transmission. Because of this, a number of studies have focused, over the past decade, in further understanding the regulation of receptor trafficking, with particular interest in the endocytosis of receptors as a mechanism for altering the strength of synapses.

$GABA_A$ receptors have been shown to undergo constitutive clathrin mediated endocytosis, and this has been shown to be a highly regulated process mediated by various associated proteins and post-translational modifications (reviewed in Kittler et al., 2002; Kittler and Moss, 2003; Arancibia-Carcamo and Moss, 2006). The internalisation of $GABA_A$ receptors has been recently shown to be regulated

by the phosphorylation state of the β subunit (Kittler et al., 2005), a mechanisms that has been shown to be important in the downregulation of GABAergic function upon D3 receptor activation in the nucleus accumbens (Chen et al., 2006). In addition, $GABA_A$ receptor endocytic sorting has been shown be regulated, in part, by the association of the $GABA_A$ receptor β subunit with HAP1 (Kittler et al., 2004b). Consistent with the suggested role of endocytosis in regulating synaptic strength, blockade of endocytosis results in an upregulation of GABAergic currents in cultured hippocampal neurons (Kittler et al., 2000, 2005). In addition, overexpression of HAP1 causes an increase in the expression of $GABA_A$ receptors at the cell surface in cultured cortical neurons and an increase in the amplitude of GABAergic currents in cultured hippocampal neurons (Kittler et al., 2004b). Furthermore this is confirmed by results from HAP1 knockout mice that show reduced levels of $GABA_A$ receptor surface expression in the hypothalamus (Sheng et al., 2006). In contrast, little is known regarding the subunit dependence of endocytic sorting or the regulatory mechanisms.

The results presented in this thesis provide further evidence to support the idea that the regulation of postsynaptic $GABA_A$ receptor cycling could be an effective means of regulating inhibitory synaptic transmission. Furthermore, it demonstrates the importance of $GABA_A$ receptor subunit composition and describes a previously uncharacterised mechanism by which only synaptic receptors are differentially regulated along the endosomal pathway. In addition, whereas regulation of endocytosis can result in dynamically regulating individual synapses, these results suggest that the regulatory step may be occuring further down the endocytic pathway probably at the level of the early endosome. Endosomes have been shown to be found along dendrites close to post-synaptic sites. However it is likely that multiple synapses share the same endocytic organelles as has been previously demonstrated for excitatory spines (Cooney et al., 2002). This suggests, that regulation of endocytic sorting can also regulate multiple synapses at the same time as observed for example in synaptic scaling (Turrigiano et al., 1998; Turrigiano and Nelson, 2000, Kilman et al., 2002).

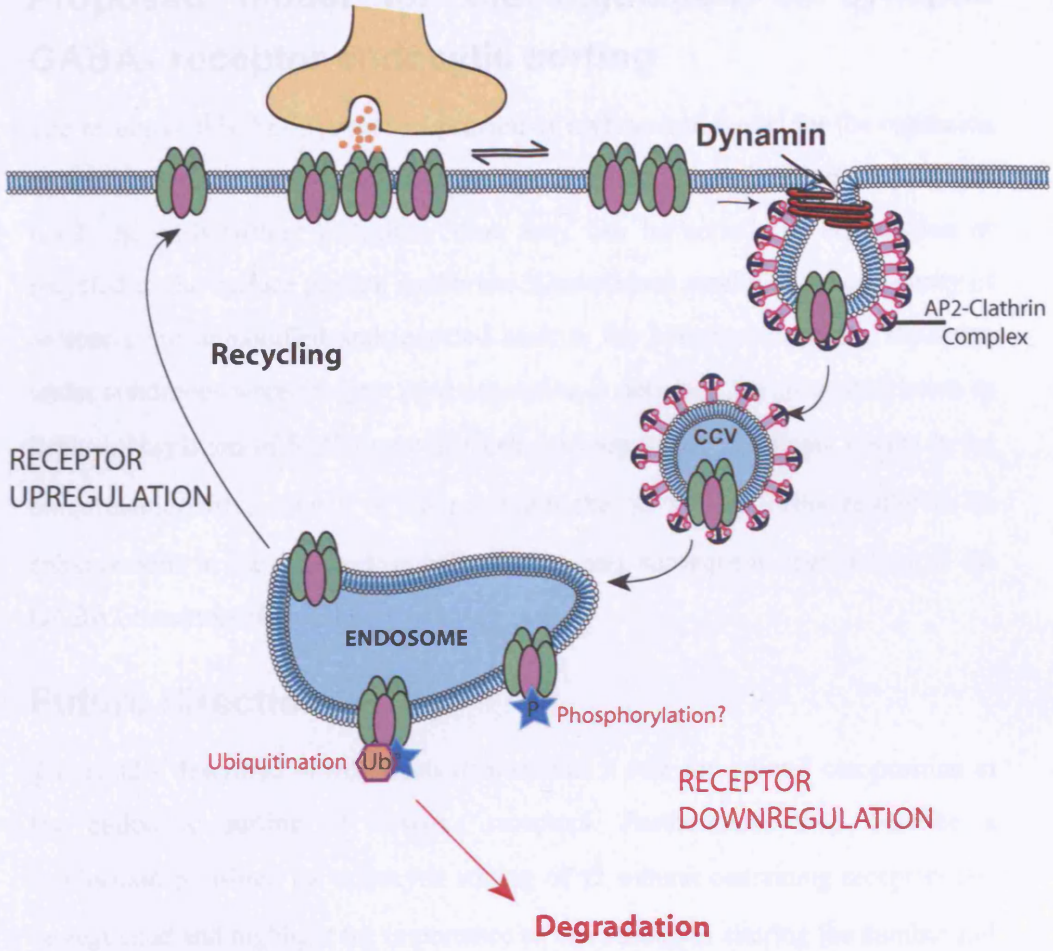


Figure 6.1: Proposed model for the regulation of $GABA_A$ receptor endocytic sorting. $GABA_A$ receptors undergo constitutive clathrin mediated endocytosis. Receptors are thought to move out of the synapse before entering clathrin coated pits. Upon internalization $GABA_A$ receptors are transported to sorting endosomes where they can be sorted for either recycling or degradation. Under conditions in which $GABA_A$ receptor function needs to be downregulated, phosphorylation or dephosphorylation of S327 in the γ2 subunit leads to ubiquitination of this subunit which results in the late endosomal targeting of $GABA_A$ receptors and subsequent degradation in the lysosome.

Proposed model for the regulation of synaptic $GABA_A$ receptor endocytic sorting

The results of this thesis propose a previously undescribed model for the regulation of inhibitory synaptic strength. Upon internalisation, synaptic $GABA_A$ receptors reach the early/sorting endosome were they can be sorted for degradation or recycled to the surface plasma membrane. Under basal conditions, the majority of receptors are unmodified and recycled back to the plasma membrane. However, under conditions were receptor downregulation is necesary, the phosphorylation or dephosphorylation of S327 in the intracellular loop of the γ2 subunit results in the ubiquitination of a stretch of lysines witin the γ2 subunit. This results in an enhancement in the late endosomal targeting and subsequent degradation of the $GABA_A$ receptors (Fig. 6.1).

Future directions

The results described in this thesis demonstrate a role for subunit composition in the endocytic sorting of $GABA_A$ receptors. Furthermore, they describe a mechanism by which the endocytic sorting of γ2 subunit containing receptors can be regulated and highlight the importance of this sorting in altering the number and size of inhibitory synapses. However, further questions need to be answered in order to fully understand this regulatory mechanism.

The importance of a serine residue (S327) within the intracellular loop of the γ2 subunit in regulating $GABA_A$ receptor ubiquitination and degradation raises the possibility of regulation by phosphorylation. S327 is a known PKC substrate although contradictory findings mean that the functional effects of phosphorylation at this site are not yet fully understood. In order to further elucidate the signalling pathways upstream from this S327 mediated ubiquitination of $GABA_A$ receptors it will be necessary to assess whether ubiquitination is regulated in a positive or negative manner by phosphorylation.

In addition, it would be interesting to identify the machinery involved in the ubiquitination and trafficking of $GABA_A$ receptors. In particular the identification of the E3 ligase involved in this process might provide important information on the physiological conditions in which $GABA_A$ receptor downregulation by this mechanism is important.

Finally, it would be interesting to analyse the effects on $GABA_A$ receptor ubiquitination of already known physiological conditions that cause a downregulation in GABAergic function. For example, NMDA receptor dependent activation of calcineurin has been shown to dephosphorylate $GABA_A$ receptors at S327 causing a reduction of $GABA_A$ receptors at the cell surface (Stellwagen et al., 2005). The link to S327 makes the model described above an attractive mechanism for explaining this process.

In addition, it is possible that the mechanism proposed, based on the results in this thesis, may play a role in the synaptic scaling of GABAergic function (Turrigiano and Nelson, 2000; Kilman et al., 2002; Stellwagen et al., 2005). The identification of the subcellular localisation at which the endocytic sorting of $GABA_A$ receptors occurs will be important to provide further information as to whether this mechanism could be important for synaptic scaling. Furthermore, the synaptic downscaling of $GABA_A$ receptors has been recently observed in response to TNF-α (Stellwagen et al., 2005). It would therefore be interesting to see whether TNF-α application or NMDA receptor activation can cause a change in the ubiquitination of $GABA_A$ receptors and whether this downregulation can be blocked by leupeptin treatment or by mutation of the stretch of lysines or S327.

7. References

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